Molecular Modeling and *In-silico* Analysis of Phenazine-1-Carboxylic Acid (*phz*) and Hydrogen Cyanide (*hcn*) Genes of Atrazine Degrading Bacteria

DISSERTATION

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Certificate

This is to certify that the research work embodied in this dissertation entitled "Molecular Modeling and In Silico Analysis of Phenazine-1-Carboxylic Acid (phz) and Hydrogen Cyanide (hcn) Genes of Atrazine Degrading Bacteria" is an original work, unless otherwise stated, carried out by ***Ms. Saema Usmani****, under my supervision, and is suitable for the award of M.Phil. degree in Microbiology of the Aligarh Muslim University, Aligarh, India.*

Prof. Javed Musarrat 25/10/08
Supervisor

Dedicated

to

My parents

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Aligarh

Saema Usmani

Abbreviations

Å	Angstrom (0.1nm)
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Base pair
C-terminus	Carboxyt erminus
Da	Dalton
DNA	Desoxyribonucleic acid
EDTA	Ehtylene Diaminetetra Acetic acid
E. coli	*Escherichia coli*
g	Gram
h	Hour
HEPES	(2-[4-(2-Hydroxyethyl)1-piperazinyl] ethane sulphonic acid
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranosid
kb	Kilo-base pair
kDa	Kilo-Dalton
kJ	Kilo-Joule
λ	Wavelength, Lambda
min	Minute
mM	Milimolar
nm	Nanometer
N-terminus	Amino terminus
PCA	Phenazine-1-carboxylate acid
PCR	Polymerase Chain Reaction
PDB	Protein data bank
RNA	Ribonucleic acid
rmsd	Root mean square deviations
rpm	Revolution per minute
RT	Room temperature
s	Second
TRIS	Tris-(hydroxymethyl)-aminomethane
U	Units
UV	Ultraviolet
V	Volt
µl	Microliter
µM	Micromolar
v/v	Volume/volume
w/v	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Amino Acids Abbreviations

Name	3-letter code	1-letter code\
Alanine	Ala	A
Argenine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Glu	E
Glutamate	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Phenylalanine	Phe	F
Methionine	Met	M
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophane	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Abstract

In this study, a bacterial strain with the inherent ability of degrading atrazine was isolated from wheat rhizosphere soil with a history of pesticide application. Out of 50 isolates, the strain SBJ1357 was specifically chosen based on its relatively higher growth efficiency and tolerance up to 500 μg ml^{-1} atrazine in mineral salt (MS) medium. The growth of SBJ1357 in MS medium supplemented with 500 μg ml^{-1} atrazine, indicates the ability of the strain to utilize atrazine as a sole source of carbon. The degradation potential of the strain SBJ1357 has been validated by kinetics of atrazine degradation in soil microcosm. The data exhibit complete disappearance of atrazine from soil within 20 days. The degradation isotherm demonstrates the time-dependent disappearance of atrazine with a rate constant as 0.173 day^{-1} following first order rate kinetics. The strain SBJ1357 has been characterized as *Pseudomonas aeruginosa* based on biochemical tests, Biolog assay and 16S rRNA gene homology and phylogenetic relationship methods. The broad spectrum antimicrobial activity exhibited by the strain demonstrated its inherent ability to inhibit the growth of phytopathogen (*Fusarium oxysporum*) and human pathogens (*Staphylococcus aureus* and *Candida albicans*) in antagonistic bioassays. The whole genome search of the *Pseudomonas aeruginosa* PAO101 strain for genes related to synthesis of antibiotic and other antimicrobial compounds revealed the presence of *phz* and *hcn* operons encoding genes for phenazine-1-carboxylic acid (PCA) and hydrogen cyanide (HCN) secondary metabolites, respectively. This study validates the role of these metabolites in the broad spectrum antimicrobial activity of the strain SBJ1357. Production of PCA and HCN by the strain has been demonstrated. The PCA production was quantitated spectrophotometrically measuring yellow color at 367 nm, developed in the benzene extract of the culture in Luria Bertani (LB) broth. The extract was also analysed by HPLC using C-18 Novapak (5μm) column with mobile phase of acetonitrile: water (70:30) at 254nm and the peak of PCA was noticed at ~9.5 min. The data revealed direct relationship of PCA production with quorum sensing (cell density) based on cross feeding experiment using *Agrobacterium tumefecians* strain A136 (Ti) (pCF218) (pCF372) indicator strain for the detection of AHL. The genetic element pCF 218 codes for TraR protein (AHL responsive transcription factor that recognize wide range of related AHLs) and TraR regulated *traI-lacZ* fused reporter is carried on pCF372. Hydrolysis of chromogenic substrate X-gal indicated the AHL dependent regulation of the PCA production. The PCA production was also found

influenced with the change in pH of the medium, incubation temperature, carbon source, amino acids and zinc sulphate. Furthermore, the strain also produces HCN as evident from the change in color of picric acid soaked filter paper from yellow to brown on King's B medium. The PCA antibiotic and HCN encoding operon *phz* (*phz*C,D,E&S) and *hcn* (*hcn* B&C) genes in strain SBJ1357 were characterized. The amplicons were cloned in pDrive cloning vector and integrity of the insert confirmed through PCR and *Eco*RI restriction analysis. The *In-Silico* analysis of PCA and HCN genes at nucleotide and translated amino acid levels was performed using bioinformatic tools. The phylogenetic analysis suggested the *hcn* and *phz* operons evolution may be parallel to whole genome history of the SBJ1357 strain. The phylogenetic analysis based on PhzC,D,E&S proteins revealed that the *phz* operon of *Pseudomonas* spp. may be laterally transferred from the ancestors (actinomycetes). The *In-Silico* secondary structure prediction and 3D structure based fold recognition study of Isochorismate hydrolase, a key enzyme in the PCA metabolic pathway encoded by *phz*D gene has also been performed. The 3D model of Isochorismate hydrolase (strain SBJ1357) was constructed by homology modeling using the X-ray crystallographic structure as a template. The constructed 3D model was verified by the molecular modeling and ramachandran plot qualities of the model assessed for the amount (%) of residues belonging to the disallowed region of the plot and deviation (A°) from the template. Additionally, the consensus functional sites were mapped on constructed model. Eventually the plant growth promoting activities of the strain SBJ1357 has also been validated based on production of IAA and inorganic phosphate solubilization. Thus, the innate capability of this novel strain SBJ1357 for parallel biodegradation, broad spectrum antimicrobial activity and plant growth promotion has a lot of significance in management of the agro-environmental, pathological and agronomic problems.

Preface

Atrazine, 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine, an organic compound consisting of an s-triazine-ring is a widely used herbicide in India and other countries including,U.S.A. This herbicide is used to check pre- and post-emergence broadleaf and grassy weeds in major crops by binding to plastoquinone-binding protein in photosystem-II, inhibiting electron transport. Atrazine and its derivatives are also used in many industrial processes, including the production of some dyes and explosives. It is also reported to cause hormone disruption in humans as well as in fish and amphibians. Recently, public health and ecological concerns have been raised about contamination of surface and ground water by atrazine and its chlorinated metabolites, due to their toxicity and potential carcinogenic effects. Microbial remediation has been proven to be one of the most cost-effective mitigation practices to remove atrazine. Mineralization of atrazine and its chlorinated metabolites, or complete cleavage of the triazine ring, in the rhizosphere has been demonstrated both under the laboratory and field conditions. A few bacterial strains, including *Pseudomonas* sp. ADP, have been isolated in the past decade from atrazine spill sites. These bacteria contains a series of genes on a self-transmissible plasmid, pADP-1, responsible for various processes of atrazine degradation resulting in complete ring cleavage and its rapid mineralization. The some degrader bacterial stains also produce an array of low molecular weight organic compounds with a wide range of biological activities. They were recognized as products of specialized metabolism and the reason for their existence though not immediately apparent, was speculated on ever since their discovery more than a century ago. 'Secondary metabolites' was the name given to these compounds by plant phytologists. These compounds are usually distinctive products of a particular group of organisms, sometimes even of a single strain and require a particular set of physiological conditions for the initiation of their synthesis. However, recently, several secondary metabolites have been discovered to play more essential roles namely, as antimicrobial agents and facilitators of root colonization in plants, in transcriptional modulation, mineral reduction and scavenging etc, all of which have far-reaching consequences not only for the well-being but also ultimately survival of the organism.

Studies on characterization of the specific atrazine degrader bacterial strains are sparse and warrant an in depth and systematic investigation. The aim of this study was to isolate the promising atrazine degrader bacterial strain from soil and their identification and

characterization using biochemical and molecular methods. The dissertation is is comprised of the following elements. The review of literature (Chapter-I) consists of four parts;. Chapter-II describes the materials and methods. Chapters-III and -IV deal with results and discussions, respectively. Finally the Chapter-V is devoted to the references.

Chapter-I

Review of Literature

Rapid industrialization and modernization around the globe have lead to an unprecedented environmental health problem owing to the production and release of considerable amounts of toxic chemicals and wastes. According to the Environmental Protection Agency (EPA) report (http://www.epa.gov/superfund/sites/phonefax/ products.htm), the United States had more than 40,000 chemical contaminated sites as of May 2004. Other industrialized countries in Western Europe possess even more contaminated sites in a comparatively smaller area (Prokop *et al.*, 2000). Some organic contaminants can persist in the environment for a long time and bring great threat to human health (Lucy *et al.*, 2004). They mainly include: total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs) coming from the exploration and consumption of fossil fuel, polychlorinated biphenyls (PCBs) widely used in the industrial process and are most degradation-resistant, and other chlorinated aromatics used as PCB replacement such as polychlorinated triphenyls (PCTs), halogenated compounds like perchloroethylene (PCE) and trichloroethylene (TCE) and pesticides like atrazine and bentazon (Saleh *et al.*, 2004).

In order to eliminate or control these noxious chemical pollutants in soils, the physical, chemical, and biological methods have been employed. Bioremediation is the application of biological processes for the cleanup of hazardous chemicals present in the environment (Gianfreda and Rao, 2004). It has obvious advantages over physico-chemical remediation methods due to several merits: cost-effectivenees, convenient, complete degradation of organic pollutants, and no collateral destruction of the site material or its indigenous flora and fauna (Timmis and Pieper, 1999). The extensive use of PGPR for the environmental remediation has emerged as a promising field.

Soil microbiota notably affects: (i) soil fertility (availability of plant nutrients) and health (suppression of soil-borne plant disease) and (ii) detoxifying ability (e.g., pesticides biodegradation) (Tiedje *et al.*, 1999; Musarrat *et. al.*, 2000). Soil microorganisms have long been regarded as ubiquitous. This view led to the common assumption that soil microbial communities are black boxes often considered as passive catalysts for degradation. The prevalence of soil microflora with innate and/or adaptive bioremediation potential contributes substantially in

reducing the pollution load. Thus, a large number of pesticides are efficiently degraded in soil, due to effective mechanisms of recruitment and assembly of diverse catabolic pathways in soil bacteria. The incessant exposure of soil microorganisms to sub-lethal doses of agrochemicals has been shown to develop the enhanced detoxification ability, and acquisition of new traits . These privileged microorganisms could be exploited for bioaugmentation of contaminated soil as a cost-effective method for bioremediation and soil health restoration *vis-à-vis* other mechanical technologies. It was suggested that the key to understanding soil functioning is the description of the composition and the biodiversity of soil microbial communities .

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) has been the most widely used herbicide over the last 30 years for nonselective weed control on industrial and non cropped land and for selective weed control in a crops as corn, sorghum, sugarcane, and pineapple. Annual use has been estimated to be approximately 403106 kg in the United States (US E.P.A., 1990). Atrazine has been classified as a moderately persistent herbicide, with half-lives ranging from several days to several months in soils (Khan *et al.,* 1981; Swain *et al.,* 1981). However, residues of both the parent compound and its derivatives have been detected in soils years after application (Shiavon, *et al.,* 1988). The primary mechanism for the dissipation of atrazine from the environment is through biological degradation. Dealkylation appears to be the first step in the degradative pathway, with the removal of the ethyl side chain generally preceding the removal of the isopropyl group. However, preferential deisopropylation of atrazine has been reported for a *Pseudomonas* sp. capable of dealkylation and dehalogenation (Behki *et al.,* 1986). The alkyl side chains contain the carbons capable of providing energy to microorganisms through oxidation (Erickson *et al.,* 1989). On the basis of the accumulation of dealkylated degradation products in soils treated with atrazine, it seems that the side chain carbon is susceptible to microbial attack. Various pure and mixed cultures that are capable of utilizing *s*-triazines as N sources have been isolated (Cook *et al.,* 1984; Hogrefe *et al.,* 1985). Biologically mediated dehalogenation has been definitively demonstrated (Mandelbaum *et al.,* 1993). Atrazine biodegradation has been extensively studied during the last 15 years and many microorganisms capable of degrading atrazine have been isolated.

Pseudomonas sp. ADP, isolated by (Mandelbaum *et al.* 1995) is the best characterized atrazine-mineralizing bacterium with fully elucidated catabolic pathway (Boundy-Mills *et al.*, 1997 de Souza *et al.*, 1995, 1998a; Martinez *et al.*, 2001; Sadowsky *et al.*, 1998). In this bacterium, the upper pathway involves three genes *atzA, atzB* and *atzC* that encoding for hydrolases that dechlorinate and dealkylate atrazine in sequential steps, forming hydroxyatrazine, N-isopropylammelide and ultimately cyanuric acid.

In addition, the third cyanuric acid amidohydrolase with different properties than those of Atz*D* and Trz*D* was recently purified and characterized from *Ralstonia basilensis* M91-3 (Stamper *et al.*, 2005). Furthermore, the hybrid pathways involving the *trzN–atzBC* (Piutti *et al.*, 2003; Rousseaux *et al.*, 2001; Sajjaphan *et al.*, 2004) or *atzABC–trzD* (Rousseaux *et al.*, 2001) gene combinations were found in pure cultures of atrazine-degrading bacteria while the *atz*ABCDEF–*trzDN*, atzBCDEF–*trz*N (Martin-Laurent *et al.*, 2006) and *atzBC–trzDN* (Smith *et al.*, 2005) gene combinations were found in atrazine-degrading communities. Although much is now known about atrazine catabolism in pure cultures, the pathways and the atrazine-degrading gene combinations harboured by bacterial communities are only poorly described. It has been postulated that mixed cultures are likely to have a larger capacity to deal with a range of metabolites which may be metabolized through lower pathway to CO2 and NH3 by another set of amidohydrolases coded by the atzD, atzE and atzF genes, respectively. All the atz genes, necessary for the complete atrazine catabolism, are located on a large plasmid pADP-1 (de Souza et al., 1998). Nearly identical atrazine-degrading genes have been demonstrated for several other Gram-negative bacteria (Bouquard et al., 1997; de Souza et al., 1998; Radosevich et al., 1995; Rousseaux et al., 2002) atrazine degrading pathway in Gram-positive Nocardioides sp. strain C190 ahs been reported based on the discovery of a novel hydrolase, designated as TrzN. This enzyme is characterized by broader substrate specificity than that of the *AtzA* from *Pseudomonas* ADP. Likewise, *trz*D, a gene coding an enzyme responsible for the s-triazine ring cleavage of cyanuric acid, was described in melamine-degrading *Pseudomonas sp.* NRRLB-12227(Karns *et al.*, 1999). *trz*D was also found in atrazine-degrading strains (Fruchey *et al.*, 2003, Rousseaux *et al.*, 2001) as well as in atrazine degrading communities (Martin-Laurent *et al.*, 2006). TrzD has 58% amino acid

sequence identity to AtzD from *Pseudomonas* ADP. In addition, the third cyanuric acid amidohydrolase with different properties than those of AtzD and TrzD was recently purified and characterized from *Ralstonia basilensis* M91-3 (Stamper *et al.*, 2005). Furthermore, the hybrid pathways involving the *trzN-atzBC* (Piutti *et al.*, 2003; Rousseaux *et al.*, 2001; Sajjaphan *et al.*, 2004) or *atz*ABC-*trz*D (Rousseaux *et al.*, 2001) gene combinations have been found in pure cultures of atrazine-degrading bacteria while the atzABCDEF–*trzDN*, atzBCDEF–trzN (Martin-Laurent *et al.*, 2006) and *atzBC–trzDN* (Smith *et al.*, 2005) gene combinations have been reported in atrazine-degrading communities. Although much is now known about atrazine catabolism in pure cultures, the pathways and the atrazine-degrading gene combinations harboured by bacterial communities are only poorly described. It has been postulated that mixed cultures are likely to have a larger capacity to deal with a range of substrates by virtue of increased catabolic capabilities (Slater and Lovatt, 1984). Therefore, microbial communities may be capable of degrading all, rather than a part, of a given compound and they appear to be more common in soils than individual species. Also cooperative metabolism has been shown to occur within a complex 8-member atrazine-degrading community with two pathways of atrazine degradation shown to be operating (Smith *et al.*, 2005).

Degradative pathway of atrazine by rhizospheric bacteria

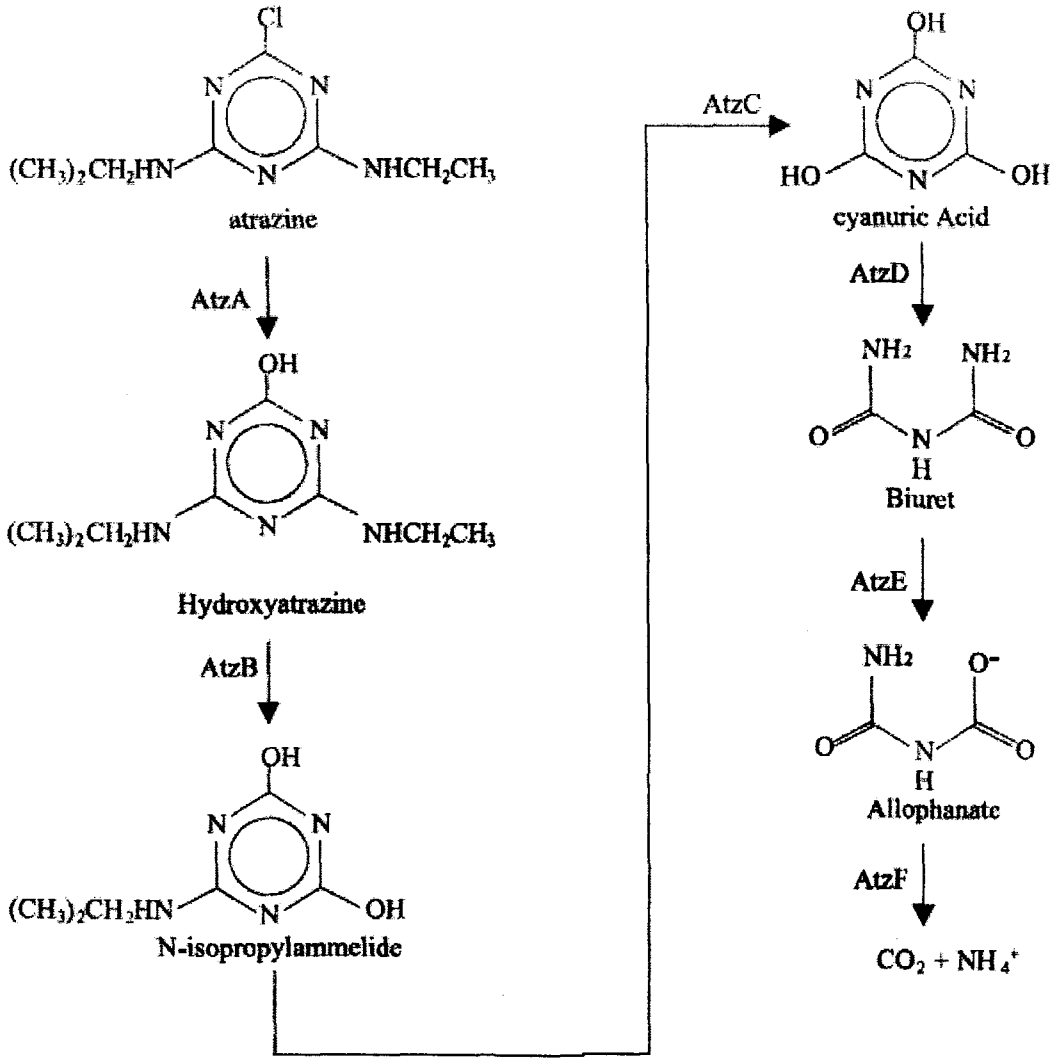


Fig. 1. Pseudomonas sp. ADP atrazine mineralisation pathway. Enzymes involved and intermediate metabolites are given. AtzA: atrazine chlorohydrolase; AtzB: hydroxyatrazine hydrolase; AtzC: N-isopropylammelide isopropylamidohydrolase; AtzD: cyanuric acid amidohydrolase; AtzE: biuret hydrolase; AtzF: allophanate hydrolase (accession number: U66917).

1.1.1. Production of siderophore

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (Loper *et al.,* 1997). Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion (Whipps *et al.,* 2001). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Loper *et al.,* 1999, O'Sullivan *et al.,* 1992). Some PGPB strains go one step further and draw iron from heterologous siderophores produced by cohabiting microorganisms (Lodewyckc *et al.,* 2002, Loper *et al.,* 1999, Raaijmakers *et al.,* 1995, Whipps *et al.,* 2001).

Siderophore biosynthesis is generally tightly regulated by iron-sensitive Fur proteins, the global regulators GacS and GacA, the sigma factors *RpoS*, PvdS, and FpvI, quorum-sensing autoinducers such as *N*-acyl homoserine lactone, and site-specific recombinases (Cornelis *et al.,* 2002, Ravel *et al.,* 2003). It has been reported that GrrA/GrrS, but not GacS/ GacA, are involved in siderophore synthesis regulation in *Serratia plymuthica* strain IC1270, suggesting that gene evolution occurred in the siderophore-producing bacteria (Ovadis *et al.,* 2004). A myriad of environmental factors can also modulate siderophores synthesis, including pH, the level of iron and the form of iron ions, the presence of other trace elements, and an adequate supply of carbon, nitrogen, and phosphorus (Duffy *et al.,* 1999).

1.1.2. Production of Hydroden cyanide

The secondary metabolite hydrogen cyanide (HCN) is produced by *Pseudomonas fluorescens* from glycine, essentially under microaerophilic conditions. The genetic basis of HCN synthesis in *P. fluorescens* CHA0 has been reported. The contiguous structural genes *hcnABC* encoding HCN synthase were expressed from the T7 promoter in *Escherichia coli*, resulting in HCN production. Analysis of the nucleotide sequence of the *hcn ABC* genes showed that each HCN synthase subunit was similar to known enzymes involved in hydrogen transfer, i.e., to formate dehydrogenase (HcnA) or amino acid oxidases (*HcnB* and *HcnC*). These

similarities and the presence of flavin adenine dinucleotide- or NAD (P)-binding motifs in HcnB and HcnC suggest that HCN synthase may act as a dehydrogenase in the reaction leading from glycine to HCN and CO_2. Hydrogen cyanide (HCN) and CO_2 are formed stoichiometrically from glycine (Castric *et al.*, 1977, Wissing *et al., 1974*) in a poorly understood oxidative reaction catalyzed by HCN synthase (castric *et al.*, 1981). This enzyme or enzyme complex appears to be membrane bound (Wissing *et al.*, 1983). In extracts, HCN synthase of a *Pseudomonas* sp. oxidizes glycine in the presence of artificial electron acceptors, e.g., phenazine methosulfate (Wissing *et al.*, 1974). Flavin adenine dinucleotide (FAD) stimulates this reaction (Wissing 1975), whereas pyrrolnitrin, an inhibitor of many flavin enzymes, and *o*-phenanthroline, an iron chelator, strongly inhibit cyanide formation *in vitro* (Wissing *et al., 1974*). HCN synthase is very sensitive to molecular oxygen and has been purified only partially from a *Pseudomonas* sp. and *P. aeruginosa* (Castric *et al.*, 1994, Wissing 1981). *In vivo*, the four electrons produced by the HCN synthase reaction are transferred to oxygen, probably by components of the respiratory electron transport chain (Castric *et al.*, 1994). In *P. aeruginosa*, no HCN is produced under fully anaerobic conditions when nitrate is the terminal electron acceptor (Castric, 1975). Optimal expression of HCN synthase occurs during the transition from the exponential to the stationary phase (Castric *et al.*, 1979) and at low oxygen levels (Castric *et al.*, 1983). Two regulatory proteins involved in these induction processes in *P. aeruginosa* have been identified: GacA and ANR (Zimmermann *et al.*, 1991). The global activator GacA, a response regulator of a two-component system, positively controls the synthesis of HCN, other secondary metabolites, and exoenzymes by a cell-density-dependent mechanism (Laville *et al.*, 1992, Reimmann *et al., 1992*). The FNR-like anaerobic regulator ANR is required for the induction of HCN synthase, the arginine deaminase pathway, and the entire denitrification pathway (Ye *et al.*, 1995, Zimmermann *et al.*, 1991). *P. aeruginosa* mutants affected in either *gacA* or *anr* produce very little HCN (Zimmermann *et al.*, 1991). *P. fluorescens* CHA0 is an aerobic, root-colonizing biocontrol bacterium that protects several plants from root diseases caused by soil borne fungi (Schnider *et al.*, 1995, Voisard *et al.*, 1994). HCN production by strain CHA0 contributes to the suppression of black root rot of tobacco, a disease caused by *Thielaviopsis basicola*, under gnotobiotic

conditions (Voisard *et al.,* 1989). GacA-negative mutants of strain CHA0, which are pleiotropically defective in the synthesis of HCN, antibiotics, and exoenzymes, have lost the ability to protect tobacco from black root rot (Laville *et al.,* 1992, Sacherer *et al.,* 1994).

1.1.3. Production of phenazines-1-carboxylic acid

Phenazines (Phz) are N-containing heterocyclic pigments. They are regarded as low-molecular-weight ("secondary") metabolites synthesized by a limited number of bacterial genera including *Pseudomonas, Burkholderia, Brevibacterium, and Streptomyces* (Elander *et al.,* 1968). Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi (Kitten *et al.,* 1998). This activity is connected with the ability of phenazine compounds to undergo oxidation-reduction transformations and thus cause the accumulation of toxic superoxide radicals in the target cells (Keel *et al.,* 1992). Some phenazine compounds can act as bacterial virulence factors. For example, pyocyanin, produced by the opportunistic pathogen *Pseudomonas aeruginosa* during cystic fibrosis, has been shown to inhibit the ciliary function of respiratory epithelial cells. Phenazine antibiotics produced by the biocontrol strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 are major factors in the ability of these strains to inhibit the growth of fungal root pathogens. Moreover, studies involving phenazine-deficient mutants have clearly demonstrated that antibiotic production in natural habitats plays an important role in the ecological competence and long-term survival of these strains in the environment (Picard *et al.,* 2000). Early studies with radiolabeled precursors revealed tight links in several microorganisms between biosynthesis of phenazine compounds and the shikimic acid pathway (Elander *et al.,* 1968). Phenazine-1,6-dicarboxylic acid is believed to be the first phenazine formed and from which others are derived . It was also proposed that the phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid and that enzymes involved in this conversion must have many features in common with anthranilate synthases. Despite intensive biochemical studies, the biosynthetic intermediates have not been identified and little is known about the genetics of phenazine synthesis.

1.1.3.1. Biosynthesis of phenazines

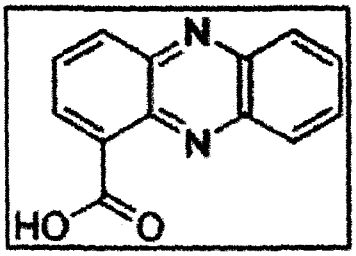


Fig 2: Structure of phenazine

The molecular backbone of phenazines consists of three aromatic rings with two nitrogen atoms in the middle ring. The phenazine biosynthetic operon contains 7 conserved genes, *phz*ABCDEFG, and is present in phenazine producing Pseudomonas strains such as, *P. fluorescens* strain 2-79 (Mavrodi *et al.*, 1998), *P. aereofaciens* strain 30-84 (Pierson, III *et al.*, 1995), *P. aeruginosa* strain PAO1 (Mavrodi *et al.*, 2001; Stover *et al.*, 2000) and P. chlororaphis strain PCL1391 (Chin-A-Woeng *et al.*, 2001). The pathway for phenazine biosynthesis branches from the shikimate pathway which is essential for the biosynthesis of aromatic amino acids (Turner and Messenger, 1986). PhzC is involved in the synthesis of shikimic acid and bypasses the DAHP (3-deoxy-D-arabino-heptulosonic acid 7-phosphate) synthetase. PhzE converts chorismate into ADIC (2-amino-2-deoxyisochorismic acid), PhzD converts ADIC into DHHA (trans-2, 3- dihydro-3-hydroxyanthranilic acid) and PhzF dimerizes two DHHA molecules into phenazine- 1-carboxylic acid (PCA) (Blankenfeldt *et al.*, 2004). PhzA and PhzB accelerate this dimerization process and PhzG reacts with the phenazine precursor and catalyzes an oxidation/aromatization reaction (Parsons *et al.*, 2004). Some strains contain additional phenazine modification genes that allow the modification of PCA into other phenazine derivatives. In *P. chlororaphis* strain PCL1391 an additional phzH gene converts PCA into PCN (Chin-A-Woeng *et al.*, 2001). In *P. aereofaciens* strain 30-84, an additional *phz*O leads to the synthesis of 2-hydroxyphenazine. In *P. aeruginosa* PhzH converts PCA into PCN and PhzM and PhzS convert PCA into pyocyanin (Mavrodi et al., 2001).

1.1.3.2. The biochemical properties of phenazines

The role and mode of action of phenazines synthesized by Pseudomonas is diverse and not well understood. Phenazines contribute to the ecological fitness of the producer strains (Mazzola *et al.,* 1992) and are thought to protect favorable niches. Plant roots colonized with phenazine producing pseudomonads benefit from this property by preventing pathogens access to the roots. Phenazines have a broad antibiotic spectrum and repress growth of microbes including fungi and Gram-positive bacteria and eukaryotes. The mode of action of phenazines is very diverse and includes intercalation with DNA, interaction with topoisomerases, anti-oxidation characteristics, and generation of free radicals (Gamage *et al.,* 2002; Laursen and Nielsen, 2004). One or a combination of these characteristics explains the growth inhibitory action of phenazines. Beside, the PCN of *P. chlororaphis* strain PCL1391 have been shown to be involved in microbial mineral reduction of crystalline iron and manganese oxide and increased the availability by dissolving these minerals. Phenazines can reduce these mineral crystals because of their redox-active characteristics and are thought the function as an electron shuttle, thereby dissolving large amounts of minerals with a small amount of PCN (Hernandez *et al.,* 2004).

1.1.3.3. Genetic diversity of phenazines

The products of the *phz* structural genes from *P. aureofaciens* strain 30-84 are similar to enzymes from the shikimic acid and tryptophan biosynthetic pathways.Two other genes, phzI and phzR, encode parts of a quorum-sensing circuit that regulates phenazine production in strain 30-84 in a cell density-dependent manner (Duffy *et al.,* 1999). Two new genes from the homologous locus of strain 30-84 are also described, and the structure and function of the biosynthetic gene clusters from the two strains are compared. Results of this study suggest that the mechanism of phenazine biosynthesis is highly conserved among fluorescent Pseudomonas species. Currently, over 50 naturally occurring Phz compounds have been described and mixtures of as many as ten different Phz derivatives can occur simultaneously in one organism (Turner *et al.,* 1986, Mavrodi *et al.,* 1998,). Growth conditions determine the number and type of Phz synthesized by an individual bacterial strain. For example, *P. fluorescens* 2-79

produces mainly PCA (phenazine 1-carboxylic acid), whereas *P. aureofaciens* 30-84 not only produces PCA but also lesser amounts of 2-OH-phenazines. The major Phz synthesized by *P. aeruginosa* is pyocyanin (1- OH-5-methyl Phz) (Weinberg *et al.*, 1970). It has been shown that bacterization of wheat seeds by *P. fluorescens* strains 30-84 and 2-79 provides primary protection against *G. graminis tritici* on account of release of Phz. Almost all Phz exhibit broad spectrum activity against bacteria and fungi. In addition to inhibiting fungal pathogenesis, Phz play an important role in microbial competition in rhizosphere, including survival and competence (Mazolla *et al.*, 1992). Use of mutants of strains 30-84 and 2-79 has confirmed their long-term survival in wheat rhizosphere on account of their ability to produce phenazine (Pierson *et al.*, 1994). The intense colour of this molecule, its antibiotic property and involvement in pathogenic reaction has made it an interesting molecule for study (Knight *et al.*, 1979). Phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid (Chang *et al.*, 1969), wherein the amide nitrogen of glutamine serves as the immediate source of N in the heterocyclic nucleus. However, it is believed that *Phz* can accept electrons, yielding a relatively stable anion radical that readily undergoes redox cycle. It includes biosynthesis of Mn-containing superoxide dismutase (Mn SOD) which causes enhanced production of superoxide radical. There is a distinct possibility that the antibiotic action of pyocyanin is actually a result of toxicity of superoxide radical and H_2O_2 produced in increased amounts in its presence (Hassan *et al.*, 1980).

1.1.3.4. Functional analysis of phenazines

Structural and functional analysis shows that seven genes, *phzABCDEFG*, are involved in the synthesis of PCA. These are localized within a 6.8 kb fragment in *P.fluorescens* 2-79. The Phz biosynthetic loci in *P. fluorescens* 2-79 (Turner *et al.*, 1986 and Mavrodi *et al.*, 1998), *P. aeruginosa* PAO1 and *P. chlororaphis* PCL 1394 are highly conserved. Each *phz* locus contains a set of seven gene core operons, regulated in a cell density-dependent manner by homologues of LuxI, and LuxR (Ching A-Woeng *et al.*, 1998 and Latifi *et al.*, 1995). In *P. fluorescens* 2-79, *P. aureofaciens* 30-84, and *P. chlororaphis* PCL 1391, the two homologues (*phzI/R*) are found directly upstream of the Phz core. Phz production in *P.*

aeruginosa is controlled by two sets of regulatory proteins, *rhlI/R* and *lasI/R* that are located elsewhere in the genome. The core gene products, PhzC, PhzD and PhzE, which are homologous with PhzE, PhzA and PhzB in strain 30-84, are similar to enzymes of shikimic acid and chorismic acid metabolism. PhzG is similar to pyridoxamine phosphate oxidase, which was found to be the source of cofactor for the PCA synthesizing enzyme(s). Products of PhzA and PhzB genes are highly homologous and appear to be involved in the stabilization of a putative PCA-synthesizing multienzyme complex. Based on the functional analysis of *phz* genes in strains 2-79 and 30-84, it has been suggested that various fluorescent pseudomonads have similar PCA-synthesizing systems (Mavrodi *et al.,* 1998, Ching A-Woeng *et al.,* 2003). Although *phz* biosynthetic loci of various strains of fluorescent pseudomonads are highly homologous, individual species differ in the range of compounds they produce. For example, *P. fluorescens*2-79 produces only PCA, whereas *P. aureofaciens* 30-84 produces, in addition to PCA, lesser amounts of 2-OHPHZ- 1-carboxylic acid (2-OH-PCA) and small quantities of 2-OH-PHZ. The conversion of PCA to 2-OH-PCA in strain 30-84 is brought about by a gene *phzO* which is located immediately downstream of the biosynthetic operon in strain 30-84. orthoposition relative to carboxyl group, which results in the synthesis of 2-OH-PCA. PhzO is a non-heme, flavindiffusible monooxygenase that adds a hydroxyl group to PCA.

1.1.3.5. Regulation of phenazine production in *Pseudomonas* by environmental factors

Biotic and abiotic environmental conditions in the rhizosphere vary substantially and are dependent on e.g. soil type, season and root exudate composition. Factors that affect the production of anti fungal metabolites (AFM) by biocontrol *Pseudomonads* include minerals, oxygen tension, temperature, pH, osmolality, phosphate, carbon and nitrogen sources, and fungal, bacterial and plant metabolites (Campa et al., 1993; Duffy and Défago, 1997; Duffy and Défago, 1999; Haas and Keel, 2003; Messenger and Turner, 1983; Ownley et al., 2003; Raaijmakers *et al.*, 2002; Slininger and Jackson, 1992; Slininger and Shea- Wilbur, 1995; van Rij et al., 2004). Lack of knowledge and variation of these conditions is thought to cause inconsistent biocontrol in the field (Handelsman and Stabb, 1996; Thomashow and

Weller, 1988). Chemical analyses of total root exudates and bacterial biosensor strains have identified environmental conditions as they occur in the rhizosphere and indicated that conditions are often nitrogen limited, oxygen limited and rich in organic acids and sugars (Haas and Keel, 2003; Højberg *et al.*, 1999; Lugtenberg and Bloemberg, 2004; Lugtenberg and Dekkers, 1999; Simons *et al.*, 1997; Vancura, 1964). Identifying conditions that favour the production of AFM could help to improve biocontrol. Production of the antifungal metabolite phenazine in *P. chlororaphis* strain PCL1391 (Van Rij *et al.*, 2004), *P. fluorescens* strain 20-79 (Slininger and Shea-Wilbur, 1995), *P. aeruginosa* strain PNA1 (Anjaiah *et al.*, 2006) and *P. aureofaciens* (Korth, 1973) is favoured by growth in rich media and by the presence of aromatic amino acids, glycerol and glucose (Kanner *et al.*, 1978). Ferric iron has a stimulating effect on phenazine production by *P. chlororaphis* strain PCL1391 (van Rij *et al.*, 2004) and *P. fluorescens* strain 2-79 (Slininger and Jackson, 1992). Fungal metabolites can suppress the production of AFM (Duffy et al., 2003). Fusaric acid, a fungal metabolite produced by *Fusarium* (Bacon *et al.*, 1996; Schouten *et al.*, 2004), represses the production of 2,4-diacylphloroglucinol in *P. fluorescens* strain CHA0 (Duffy and Défago, 1997) and of PCN in *P. chlororaphis* strain PCL1391 (van Rij *et al.*, 2005). Soils with high zinc concentrations repress the production of fusaric acid by *Fusarium oxysporum* f. sp. *radicis-lycopersici* and improve biocontrol (Duffy and Défago, 1997).

1.1.3.6. Genetic regulation of phenazines in pseudomonads

1.2.3.6.1. Global regulators *gacS* and *gacA*

The two component regulatory system *gacS/gacA* (global antibiotic and cyanide Sensor/Activator) is highly conserved in pseudomonads and regulates many traits including virulence, survival, motility, biofilm formation, phase variation and the production of extracellular products (Chancey *et al.*, 2002; Chatterjee *et al.*, 2003; Chin-A-Woeng *et al.*, 2005; de Souza *et al.*, 2003; Heeb and Haas, 2001; Parkins *et al.*, 2001; van den Broek *et al.*, 2005). In *Pseudomonas* biocontrol strains, GacS and GacA are essential for the production of at least some secondary metabolites and extra-cellular enzymes with anti-microbial activity including phenazines, proteases, HCN, diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, chitinase, phospholipase C, all of which can be involved in biocontrol (Chancey *et*

al., 1999; Chin-A-Woeng *et al.*, 2005; Heeb and Haas, 2001; Zuber *et al.*, 2003). GacS is a histidine kinase sensor located in the cytoplasmic membrane. GacA is a transcriptional regulator and can be activated through phosphorylation by GacS. Between the global regulators *gacS/gacA* and production of secondary metabolites several molecular intermediates are known which include small non coding regulatory RNAs, *psrA*, *rpoS*, and the quorum sensing regulators *phzI/phzR*

1.2.3.6.2. The molecular regulators *PsrA* and *RpoS*

The regulators *psrA* (*Pseudomonas* sigma regulator) and *rpoS* (stationary phase alternative sigma factor σs) affect the antibiotic production of in *P. fluorescens* strain Pf-5 (Sarniguet et al., 1995), *P. aeruginosa* strain PAO1 (Schuster *et al.*, 2004; Suh *et al.*, 1999) and P. chlororaphis strain PCL1391 (Chin-A-Woeng et al., 2005; Girard et al., 2006). *PsrA* acts as a positive regulator of *rpoS* gene expression and *RpoS* protein levels, which places *rpoS* downstream of *psrA* (Kojic and Venturi, 2001); (Girard *et al.*, 2006). *PsrA* is a transcriptional activator that contains a typical helix-turn-helix DNA binding motif (Chin-AWoeng *et al.*, 2005; Girard *et al.*, 2006; Kojic and Venturi, 2001). Sigma factors, including *RpoS*, determine the specificity of the RNA polymerase. *RpoS* controls the expression of genes involved in responses to various stress conditions, for example starvation. The expression of *psrA* and *rpoS* is dependant on gacS/gacA and places *psrA/rpoS* downstream of gacS/gacA in the regulatory cascade of PCN biosynthesis (Chin-A-Woeng *et al.*, 2005; Whistler *et al.*, 1998).

1.2.3.6.3. Post transcriptional regulation by RsmA and small non-coding RNAs

RNA binding proteins and small non-coding regulatory RNAs regulate the production of secondary metabolites and exo-enzymes in *Pseudomonas* spp. The translational repressor RNA binding protein RsmA represses the production of pyocyanin and N-acyl-homoserine lactones in *P. aeruginosa* strain PAO1 (Pessi *et al.*, 2001). The biocontrol strain *P. fluorescens* strain CHA0 contains two RNA binding proteins, RsmA and its homolog RsmE, both repressing the production of hydrogen cyanide, exoprotease, and 2,4-diacylphloroglucinol (Blumer *et al.*, 1999; Kay *et al.*, 2005; Reimmann *et al.*, 2005). RsmA like proteins are presumed to

interact with the ribosome binding sites of target genes and prevent their transcription. Genome sequence analyses of different sequenced pseudomonads predict that the number of rsmA homologues varies between different Pseudomonas strains from one to six (Reimmann *et al.*, 2005). A rsmA homologue was also identified in *P. chlororaphis* strain PCL1391 (G. Girard, personal communication). Repression by RsmA is relieved by GacS/GacA controlled small non-coding RNAs such as PrrB in *P. fluorescens* strain F113 (Aarons *et al.*, 2000), RsmZ and RsmY in *P. fluorescens* strain CHA0 (Heeb et al., 2002; Valverde et al., 2003), and RsmZ and RsmB in *P. aeruginosa* strain PAO1 (Burrowes *et al.*, 2005; Heurlier *et al.*, 2004). These small non-coding RNAs bind to RsmA, or its homologues, and relieve the repression of the synthesis of secondary metabolites and exo-enzymes. The observation that rsmA is found in *P. chlororaphis* strain PCL1391 suggests that a similar post transcriptional mechanism occurs in this strain.

1.2.3.6.4. Quorum sensing

Several genetic and biochemical studies in the 1960s and 70s, provided compelling evidence for an 'organized social behaviour' employing sophisticated communication systems to coordinate the activities of individuals within a population. Once considered to be a rare phenomenon, restricted to a few scattered examples, it is now increasingly apparent that an extensive range of microorganisms have the ability to perceive and respond to the presence of neighbouring populations. The term 'quorum-sensing' has been employed to describe such density-dependent phenomenon. Such a system is accomplished by the extracellular accumulation of small, self-generated chemical signaling moieties that induce a concerted effort on behalf of a population to produce the desired phenotypic effect. The term quorum-sensing reflects the minimum threshold level of individual cell mass required to initiate a concerted population response. The signal molecule used for communication was dubbed as 'autoinducer', owing to its origin inside the bacterial cell. The desired is obtained arrived at by attainment of quorum employing the autoinducer and the process was labelled as 'autoinduction'. In other words, the whole circuit relies on the intracellular production and export of a low-molecular mass signaling molecule, the

extracellular concentration of which grows with the population density of the producing organism. The signaling molecule can be sensed and re-imported into these cells, thus allowing the whole population to respond to changing environment/requirement once a critical concentration (corresponding to a particular cell density) has been achieved. Several classes of microbially-derived signaling molecules have now been identified. Broadly, these can be divided into two main categories (i) amino acids and short peptide derivatives, commonly utilized by Gram-positive bacteria3,4, and (ii) fatty acid derivatives, called homoserine lactones (HSLs) frequently utilized by Gram-negative members 5,6. Whatever may be the nature of the signal molecule, the whole network functions by its re entry into the cell either via diffusion or an active transport 6. The signaling mechanism involves subsequent interaction of the signal with an intracellular effector that will induce the pathway for the concerned phenotype.

1. 3. Plant growth promoting factors

1.3.1. Production of Indole acetic acid (IAA)

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Glick *et al.,* 1995). Frankenberger and Arshad, (1995) have discussed in detail the role of auxins, cytokinins, gibberellins, ethylene and absicisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins has been reviewed by various authors over the last 20 years (Pilet *et al.*, 1979). The production of an active substance by the fungi *Rhizopus suinus* and *Absidia ramosa* was identified to be auxin. Several studies have reported the production of indole-3-acetic acid by microorganisms in the presence of the precursor tryptophan or peptone. Some of the plant responses to auxin are as follows: a) cell enlargement; b) cell division; c) root initiation; d) root growth inhibition; e) increased growth rate; f) phototropism; g) geotropism; and h) apical dominance. Eighty percent of microorganisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites (Kampert *et al.,* 1975; Loper and Schroth, 1986). Bacteria belonging to the genera *Azospirillum*, *Pseudomonas, Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus*

and *Bradyrhizobium japonicum* have been shown to produce auxins, which help in stimulating plant growth (Patten and Glick, 1996). Various metabolic pathways such as a) indole-3-acetamide pathway; b) indole-3-pyruvic acid pathway; c) tryptophan side chain pathway; d) tryptamine pathway; and e) indole-3-acetonitrile pathway are involved in the production of IAA. Phytopathogens such as *Agrobacterium tumefaciens, A. rhizogenes* and *P. syringae* pv. savastanoi synthesize IAA via the indole-3-acetamide pathway (Liu *et al.*,, 1982; Offringa *et. al.*, 1986). It has been suggested that *E. cloacae*, isolated from the rhizosphere of cucumber, synthesized IAA via the indolepyruvic acid pathway and promoted growth of various agricultural plants. Also, *P. fluorescens* demonstrated the ability to convert L-tryptophan directly into indole-3-ac*et al.*, dehyde (Narumiya *et al.*, 1979). Strains such as *B. cereus* and *A. brasilense* produced IAA by the tryptamine pathway (Perley and Stowe, 1966; Hartmann *et al.*, 1983). Bacterial production of IAA suggests that the pathways involved in IAA production may play an important role in defining the effect of the bacterium on the plant (Patten and Glick, 1996). Most of the pathogenic strains of bacteria synthesized IAA via the indoleacetamide pathway while plants use the indolepyruvic acid pathway. This helps the bacteria to evade plant regulatory signals and thus the IAA produced, induces uncontrolled growth in plant tissues. In contrast, the beneficial bacteria such as PGPR synthesize IAA via the indole pyruvic acid pathway and the IAA secreted is thought to be strictly regulated by the plant regulatory signals. Differences in the production of IAA among bacterial strains can be attributed to the various biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions (Patten and Glick, 1996).

1.3.2. Solubilization of the inorganic phosphate

The plant growth-promoting bacteria also promote the growth of plant by the mineralization of organic phosphate or by solubilization of inorganic phosphate with production of acids (Lifshitz *et al.*, 1987). These bacteria are referred to as phosphobacteria and have been considered to have potential use as inoculants showed that mixed populations of rhizosphere bacteria enhance uptake of phosphate by young barley seedlings, while causing a decrease in the uptake of

phosphorus in older plants. The increase in dry weight was directly correlated to phosphorus uptake by barley seedlings. Similar results have been reported by Lifshitz *et al.* (1987) when 1mM phosphorus was added to the growth medium, root elongation of inoculated and non-inoculated canola seedlings in sterile growth pouches. The combined effects of bacterial inoculation (*P. putida* GR12-2) and addition of phosphate on root and shoot elongation and on root and shoot weights were significant. Seed inoculation with *P. putida* GR12-2 increased the uptake of labeled phosphorus (^{32}P) and also enhanced shoot elongation of seedlings grown in sterile soil. Cattelan *et al.* (1999) performed an *in vitro* screening of 116 isolates obtained from soil for various PGPR traits including the ability of bacteria to solubilize phosphorus. The study indicated that isolates, which have the ability to produce ACC deaminase or siderophores or those able to solubilize phosphorus, might increase early soybean growth in non-sterile soil.

1.4. Application of bioinformatics analysis tools

1.4.1. Phylogenetic analysis

Studies in molecular taxonomy based on genetic comparisons have evolved the whole new field of molecular phylogeny, which is based on genetic comparisons of DNA and/or protein sequences. Phylogenetics is the study of evolutionary relationships. The evolutionary history inferred from phylogenetic analysis is usually depicted as branching, tree like diagrams that represent an estimated pedigree of the inherited relationships among molecules ("gene tree") organisms, or both. Phylogenetic is also called as "cladistics" derived from the greek word which means branch or a "clade" a set of descendants from a single ancestor. However, cladistics is a particular method of hypothesizing about evolutionary relationships. The resulting relationships from cladistic analysis and some of the basic elements are represented by a phylogenetic tree.

1.4.1.1. Construction of phylogenetic tree

The phylogenetic data analysis involves following important steps specified as under;

1. Alignment (building the data model and extracting a phylogenetic dataset)
2. Tree building

3. Tree evaluation

1.4.1.1.1. Alignment of sequences

Sequences alignment is the procedure of comparing two (pair-wise alignments) or more (multiple sequence alignment) sequence by searching for a series of individual character or character patterns that are in same order in the sequences. Two sequences are aligned by writing them across a page in two rows. Identical or similar characters are placed in same column or a mismatch or opposite a gap in the other sequence. In an optical alignment that is global and local. Sequences that are quit similar and approximately of the same length are suitable candidates for global alignment. However, in local alignment, the stretches of sequence with the highest density of matches are aligned, which generates one or islands of matches or sub-alignments in the aligned sequences. Phylogenetic sequence data usually consist of multiple sequence alignments.the individually aligned-base positions are commonly referred to as sites. These sites are equivalent to characters in theoretical phylogenetic discussion, and the actual base (or gap) occupying a site is referred as the character state. A typical alignment procedure involves application of a programme such as ClustalW, followed by manual alignment, editing and submission to a tree building programme. The guide tree from ClustalW is formed as a phylip tree file and can be imported in various tree-drawing programmes. Some programmes are designed to simultaneously optimize an alignment and a phylogenetic tree (e.g tree align and malign). Alignment parameters vary dynamically with evolutionary divergence (Thompson et al., 1994), and therefore more base mismatches as the sequences become more divergent. Thus, the alignment parameters should also be adjusted to prevent closely related, over represented sequences (Thompson et al., 1994: Hughey et al., 1996). This is accomplished by down weighting the alignment score contribution of closely related sequences. These dynamic parameter adjustments are both implemented in ClustalW, whereas sequence weighing is implemented in SAM (alignment programme). The following points should be considered when constructing a multiple sequence alignment for a phylogenetic analysis

(1) The alignment step in phylogenetic analysis is one of the most important steps because it produces the data set, on which models of evolution are based.

(2) It is not uncommon to edit the alignment, deleting unambiguously aligned regions and inserting or deleting gaps to more accurately reflected evolutionary processes, which led to the divergence between sequences.

(3) It is useful to perform phylogenetic analysis based on a series of slightly modified alignments to determine how ambiguous regions in the alignment affect the results.

1.4.1.1.2. Tree-building methods

Tree-building methods implemented in available software are discussed in detail in the literature (Saitou, 1996; swofford et al., 1996; Li, 1997) tree building could be done using distance-based vs character based methods. Much of the discussion in molecular phylogenetic dwells on utility of distance and character based methods (Saitou, 1996; Li, 1997). Distance methods compute pair wise distances according to some measures and then discard the actual data patterns for each character .pairwise distances are therefore, not fixed as they are determined by the tree topology. The most commonly applied distance-based methods include neighbor-joining and the Fitch-Margoliash method, and the most common character-based methods include maximum parsimony and maximum likelihood.

1.4.1.1.2. 1. Distance based methods

Distance based methods use the amount of dissimilarity (the distance) between two aligned sequences to derive trees. A distance method would reconstruct the true tree if all genetic divergence events were accurately recorded in the sequence (Swofford *et al.,* 1996). Distance methods are much less computationally intensive than maximum likelihood but can employ the same models of sequence evolution. This is their biggest advantage. The disadvantage is that the actual character data are discarded. The most commonly applied distance-based methods are the unweighted pair group method with arithmetic mean (UPGMA) neighboring joining (NJ) and methods that optimize the additively of a distance tree, including the minimum evolution (ME) method.

1.4.1.1.2. 1.1. Unweighted Pair-group method with arithmetic mean (UPGMA)

UPGMA is a clustering or phenotypic algorithm. It joins tree branches based on the criterion of greatest similarity among pairs and averages of joined pairs. It is not strictly an evolutionary distance method (Li, 1997). UPGMA is expected to generate an accurate topology with true branch lengths only when the divergence is according to a molecular clock (Swofford *et al.*, 1996) or approximately equal to raw sequence dissimilarity.

1.4.1.1.2.1.2. Neighbor joining

Neighbor-joining algorithm is commonly applied with distance tree building, regardless of the optimization criterion. The fully resolved tree is "decomposed" from a fully unresolved "star" tree by successively inserting branches between a pair of closest neighbors and the remaining terminals in the tree. The closest neighbor pair is then consolidated, effectively reforming a star tree, and the process is repeated. The method is comparatively rapid.

1.4.1.1.2. 1.3. Fitch-margoliash (FM)

The Fitch-Margoliash (FM) method seek to maximize the fit of the observed pair wise distances to a tree by maximizing the squared deviation of all possible observed distances relative to all possible path lengths on the tree (Felsentein,1997). There are several variations that differ in how the error is weighted. The variance estimates are not completely independent because the errors in all the internal tree branches are counted at least twice (Rzhetsky and Nel, 1992)

1.4.1.1.2.1.4. Minimum evolution (ME)

Minimum evolution seeks to find the shortest tree that is consistent with the path lengths measured in a manner similar to FM. It works by minimizing the squared deviation of observed to tree-based distances (Rzhetsky and Nei,1992; Swofford et al., 1996; Felsenstein,1997). Unlike FM, it does not use all possible pair wise distances and pair wise distances and associated tree path lengths. However, it fixes the location of internal tree nodes based on the distances to external nodes

and than optimizes the internal branch length according to the minimum measured error between these observed points. It thus purports to eliminate the non independences of FM measurements.

1.4.1.1.2. 2. Character based methods

The character-based have little in common with each other, besides the use of the character data at all steps in the analysis. This allows the assessment of the reliability of each base position in the alignment on the basis of all other base positions. The two common character-based methods are discussed below:

1.4.1.1.2. 2.1. Maximum parsimony (MP)

Maximum parsimony is an optimization criterion that adheres to the principle that the data explanation should be simple with few ad hoc assumptions. Thus, the tree generated with method is short. To accommodate substitution bias MP is amenable to weighting. However, the method performs poorly when there is substantial among-site rate heterogeneity (Huelsenbeck, 1995).MP analysis tend to yield numerous trees that have the same score.

1.4.1.1.2. 2. 2. Maximum likelihood:

(ML) turns the phylogenetic problem inside out. ML searches for the evolutionary model, including the tree itself that has the highest likelihood of producing the observed data. In the practice, ML is derived for each base position in an alignment. The likelihood is calculated in terms of the probability that the pattern of variation at a site would be produced by a particular substitution process, given a particular tree and overall observed base frequencies. The likelihood becomes the sum of the probilities of each possible reconstruction of substitutions under a particular substitution process. The likelihoods for all the sites are multiplies to give an overall likelihood of the tree. Since ML uses great amounts of computational times, it is usually impractical to perform a complete search that simultaneously optimizes the substitution model and the tree for a given data.

1.5. Molecular modeling

The genome-sequencing projects are providing a detailed "parts list" of life. A key to understanding this list understands the function of each gene and each protein at various levels (Skolnick and Fetrow, 2000). Structure and function in proteins are closely related. Despite rapid growth of known protein sequences, direct experimental determination of their structure by nuclear magnetic ressonance (NMR) or X-ray crystallography is still quite time consuming and often limited by the protein size (NMR) or the availability of crystals (Dandekar and König, 1997). Knowledge of protein structure is fundamental to know mechanism of action, and prediction of structure for new sequences is of great value to such studies (Westhead and Thornton, 1998). When considering whole parasite genomes, comprising thousands of genes, the actual challenge is to assemble, catalogue and analyze this information in a robust and useful manner (Fairlamb, 2001). In this work, current public available bioinformatics and molecular modeling tools in proteomic domains have been used in a generic approach to determine the structure-function relationship of unknown genes. This methodology was applied to study a genomic sequence from *Leishmania amazonensis* which has been shown to have high homology to the poly-A binding protein class and for this reason here after named LaPABP.

Chapter-II

Materials & Methods

2.1. Materials

2.1.1. Experimental bacterial strain

Atrazine degrading bacterial strain SBJ1357 was isolated from the wheat rhizosphere soil.

2.1.2. Special laboratory instruments and materials

2.1.2.1. Instruments

Instruments	Made
Bacterial incubator	NSW, Pvt. Ltd. New Delhi, India
Bacterial shaker	REMI Instrument Ltd., Mumbai, India
ABI PRISM 373DNA Sequencer	Applied Biosystems, USA
Electrophoresis power supply EPS600	Biotech, R&D Laboratory, Yercaud, India
Gel documentation system	UVP, Upland, CA, USA
Heating block	Genie, Bangalore, India
Mercury thermometer	Hicks India, Ltd.
Horizontal electrophoresis unit	Genie, Bangalore, India
HPLC	Water, 2487 dual λ absorbance detector USA
pH meter	Elico, India
Balance	Anamed, India
Spectrophotometer	GBC Cintra 10e , Australia
PCR machine	TechGene Cambridge Ltd.UK
Vortex	Biosan
Water bath	Ratek instruments Pvt. Ltd. Boronia
Centrifuge C24	REMI Instrument Ltd., Mumbai, India

2.1.2.2. Chemical reagents and general materials.

Chemicals reagents	Purchased from
Agar $(C_{12}H_{18}O_9)_n$	HiMedia, Mumbai, India
Agarose, Ultra pure	Sigma Chemical Co. USA
Ammonium acetate($CH_3.COOK.NH_4$)	Sigma Chemical Co. USA
Ammonium sulfate (NH_4SO_4)	Sigma Chemical Co. USA
Ampicillin ($C_{16}H_{18}N_3O_4SNa$)	Sigma Chemical Co. USA
Acetic acid glacial ($CH_3.COOH$)	Qualigens, Mumbai, India
Benzene(C_6H_6)	Qualigens, Mumbai, India
Boric acid (H_3BO_3)	Sigma Chemical Co. USA
n-Butyl alcohol {$(CH_3.(CH_2)_3.OH$}	Merck, Mumbai, India
Calcium chloride ($CaCl_2$)	Sigma Chemical Co. USA
Chloroform ($CHCl_3$)	Merck, Mumbai, India
dNTPs	Fermentas GMBH, Germany
Dimethyl Sulphoxide (DMSO) ($CH_3.SO.CH_3$)	Sigma Chemical Co. USA
DTT ($C_4H_{10}O_2S_2$)	Sigma Chemical Co. USA
Ethanol (C_2H_5OH)	Merck, Mumbai, India
Ethidium bromide ($C_{21}H_{20}BrN_3$)	Sigma Chemical Co. USA
Glycerol ($CH_2OH.CHOH.CH_2OH$)	Sigma Chemical Co. USA
Glycine ($C_2H_5NO_2$)	Sigma Chemical Co. USA
HDTMA ($C_{19}H_{22}BrN$)	Sigma Chemical Co. USA
Hydrochloric acid (HCl)	Qualigens, Mumbai, India
IPTG ($C_9H_{18}O_5S$)	Sigma Chemical Co. USA
Iso-amyl alcohol {$(CH_3)_2CHCH_2CH_2OH$}	Qualigens, Mumbai, India
Iso-propyl alcohol {$(CH_3)_2.CHOH$}	Merck, Mumbai, India

Kanamycin ($C_{18}H_{36}N_4O_{11}.H_2SO_4$)	Sigma Chemical Co. USA
Methanol (CH_3OH)	Qualigens, Mumbai, India
β-Mercaptoethanol ($HS.CH_2CH_2OH$)	Sigma, Chemical Co. USA
Phenol (C_6H_5OH)	Merck ,Mumbai, India
Phenol/Chloroform (TE saturated)	Qualigens, Mumbai, India
Potassium acetate ($CH_3.COOK$)	Sigma Chemical Co. USA
Potassium chloride (KCl)	Sigma Chemical Co. USA
Potassium dihydrogen phosphate (KH_2PO4)	Sigma Chemical Co. USA
Phosphoric acid (H_3PO_4)	Qualigens, Mumbai, India
SDS $\{CH_3(CH_2)_{11}OSO_3Na\}$	Qualigens, Mumbai, India
Sodium acetate (CH_3COONa)	Sigma Chemical Co. USA
Sodium bicarbonate ($NaHCO_3$)	Sigma Chemical Co. USA
Sodium citrate $\{HOC(COONa)(CH_2COOH)_2\}$	HiMedia, Mumbai, India
Sodium dihydrogen phosphate (NaH_2PO4)	Sigma Chemical Co. USA
Sodium hydrogen phosphate (NaHPO4)	Sigma Chemical Co. USA
Sodium hydroxide (NaOH)	Sigma Chemical Co. USA
Sucrose (Ultra pure) ($C_{12}H_{22}O_{11}$)	Sigma Chemical Co. USA
Tryptone	HiMedia, Mumbai, India
Tetracycline ($C_{22}H_{24}N_2O_8$)	Sigma Chemical Co. USA
Tris- hydroxymethyl methylamine $\{NH_2.C(CH_2OH)_3\}$	Sigma Chemical Co. USA
X-gal ($C_{14}H_{15}Br\ ClNO_6$)	Sigma Chemical Co. USA
Xylene cyanol ($C_{25}H_{27}N_2O_6S_2Na$)	Sigma Chemical Co. USA
Yeast extract	HiMedia, Mumbai, India

Note. Other chemicals and reagents not mention in this list were purchased from the Hi-Media and Qualigens.

2.1.2.3. Plasmid cloning vectors

pGEM-T	Amp^r (Promega, Madison WI, USA)
pDrive	Amp^r and Kan^r (Qiagen Inc. USA)

2.1.2.4. Primers

Primers and oligonucleotides used in the molecular cloning and sequencing are listed in the following Table. HPLC purified oligonucleotides were obtained from Sigma Genosys, Bangalore, India.

Primers Name	Sequence (5'-3')	Tm	%GC	Length	Target genes in operons	Amplicon size (bp)
PCAf	GGCGACATGGTCAACGG	66.6	64.71	17	*Phz*C&D	1405
PCAr	CGGCTGGCGGCGTATTC	70.1	70.59	17		
PCJM2f	TCGACGCCTACTCCAACGA	66.9	57.89	19	*Phz*DE	1480
PCJM2r	CTGCTTGGCGATCATCGA	66.4	55.56	18		
PCJM3f	CAAGGTCACGCTGCTGGA	66.6	61.11	18	*phz*S& transporter protein	1499
PCJM3r	TGATCGGCTCGCTGGCTA	68.3	61.11	18		
Acaf	ACTGCCAGGGGC GGA TGT GC	75.8	70.00	20	*hcn*B&C	586
Acbr	ACGATGTGCTCGGCGTAC	65.1	61.11	18		
fD1	AGAGTTTGATCCTGGCTCAG	61.0	50.00	20	16S rRNA	1498
rD1	AAGGAGGTGATCCAGCC	60.2	58.82	17		
M13 f	GTAAAACGACGGCCAGT	-	-	-	Sequencing	-
M13 r	AACAGCTATGACCATG	-	-	-		-
SP 6	ATTTAGGTGACACTATAG	-	-	-	Sequencing	-

2.1.2.5. Enzymes

Taq polymerase	Fermentas GMBH, Germany
Lysozyme	Sigma Chemical Co., USA
RNase A	Fermentas GMBH, Germany
Restriction enzymes	Fermentas GMBH, Germany
Proteinase K	Sigma Chemical Co., USA

2.1.2.6. DNA and protein molecular weight markers

ΦX174/*BsuR* I(*Hae* III)	Fermentas GMBH, Germany
Lambda DNA/*EcoR* I+*Hind* III	Fermentas GMBH, Germany

2.1.2.7. Other reagents and kits

PCR product cloning kits	Qiagen Inc., USA.
DNA extraction kit	Qiagen Inc., USA.

LB medium		
Tryptone	10g	1.0% (w/v)
Yeast extract	5g	0.5% (w/v)
NaCl	10g	1.0% (w/v)
H_2O	1 liter	
Adjust pH to 7.0.		
Low salt LB medium		
Tryptone	10g	1.0% (w/v)
Yeast extract	5g	0.5% (w/v)
NaCl	5g	0.5% (w/v)
H_2O	1 liter	
Adjust pH to 7.0		
SOB medium		
Tryptone	20g	2.0% (w/v)
Yeast extract	5g	0.5% (w/v)
NaCl	0.5g	0.05% (w/v)

250mM KCl	10ml	2.5mM
H_2O	900ml	
Autoclave, cool to room temperature and add 10ml of sterile solution of 1M $MgCl_2$ before use.		10mM
SOC medium		

10X TBE (Tris-borate-EDTA) electrophoresis buffer		
Tris base	108g	90mM
Boric acid	55g	90mM
0.5M EDTA (pH 8.0)	40ml	2mM
H_2O	1 liter	
TE (Tris-EDTA) Buffer, pH 7.4, 7.6 or 8.0		
1M Tris, pH 7.4, 7.6, 8.0	10ml	10mM
0.5M EDTA (pH 8.0)	2ml	1mM
H_2O	1 liter	

2.2. Methods

2.2.1. Collection of soil sample

Soil samples were collected from the agricultural farm of U.P Agriculture Department, Kalai, Aligarh. Composite samples were collected and transferred to polythene bags and immediately transported to the laboratory.

2.2.2. Assessment of physio-chemical characteristics of collected soil

Physio-chemical characteristics of soil were studied using standard methods as described below:

2.2.2.1. pH

Suspension of soil sample was prepared in MQ water in the ratio of 1:2 (w/v) and allowed to stand for 30 min with continuous shaking on rotatory shaker at 100 rpm, and pH was determined using digital pH meter.

2.2.2.2. Temperature

The soil temperature was recorded at the sampling site using Hicks mercury thermometer.

2.2.2.3. Water holding capacity (WHC)

WHC of soil samples was determined according to the standard method. 50 g of air-dried soil was taken in a funnel lined with glass wool at its neck and the end fitted with rubber tubing and stop cork. 50 ml water was added to the soil and allowed to stand for 30 min. and than allowed to drain for 30 min. The difference in the volume of water initially added and the volume of water drained from the soil was taken as the WHC and expressed as percentage.

2.2.3. Isolation of atrazine degrading bacteria from the collected soil sample

A bacterium capable of degrading atrazine was isolated from agricultural soil following the enrichment culture technique. In brief, soil homogenate (1 ml) was added to 50 ml nutrient broth (NB) containing 100 μg ml^{-1} atrazine (99.0 % pure), and incubated at 30 ^{0}C with constant shaking at 150 rpm. An aliquot of 0.2 ml of the culture was plated on mineral salt (MS) medium (Appendix 1) supplemented with 500 μg ml^{-1} atrazine as a sole carbon source. The resulting colonies on MS medium were repeatedly sub-cultured on the same medium in the presence of 1000 μg ml^{-1} atrazine to confirm their degrading ability. The selected isolate SBJ1357 was identified based on colony morphology, biochemical characteristics following Bergey's Manual of Determinative Bacteriology, Biolog and 16S rRNA nucleotide sequences.

2.2.4. Kinetics of biodegradation of atrazine by SBJ1357

Briefly, aliquots of 1 kg sandy loam soil in earthen pots obtained from experimental fields of Faculty of Agricultural Sciences, AMU, Aligarh were amended with 10 mg kg^{-1}concentration of atrazine. The soil samples were enriched with freshly grown culture of the isolate SBJ1357 at a cell density of 10^7 cfu g^{-1} soil. Parallel controls consisting of sterilized soil with identical amounts of atrazine were run. At different time points between 0, 4, 8, 12, 16 and 30 days, aliquots of

15 g soil were removed in duplicate from the microcosms. The samples were subjected to organic solvent extraction three times with high performance liquid chromatography (HPLC) grade ethylacetate. The extract was filtered and evaporated to near dryness. The residues were dissolved in 5 ml of methanol and stored at 4 ^{0}C until analysis. The atrazine analysis was performed on Waters HPLC System coupled with UV/Visible detector. The separation was achieved using C-18 Novapak (5μm) column with mobile phase of acetonitrile : water (70:30) at 254 nm. The flow rate was 1.0 ml min^{-1} and absorbance was read at 254 nm. The degradation rate constant (k) was determined using the algorithm $C_t/C_o = e^{-kt}$, where C_o is the amount of atrazine in the soil at time zero, C_t is the amount of atrazine in soil at time t, and k and t are the rate constant (day^{-1}) and degradation period in days, respectively.

2.2.5. Biocontrol efficacy of the SBJ1357 against *Fusarium oxysporum*

The antagonistic ability of the strain SBJ1357 was tested, against phytopathogen (*Fusarium oxysporum*) and human pathogen (*Candida albicans*) by zone of inhibition bioassay method. In brief, the broth culture of the selected pathogens (10^5 CFU ml^{-1}) was plated on the surface of potato dextrose agar (PDA) (Appendix 2). A small wells (4 mm x 2 mm) cut in these agar plates, and 0.1 ml of 1x 10^8 CFU ml^{-1} test strain culture was added. Antimicrobial activity of test strain against the pathogens was evaluated on the basis of zone of inhibition after 3 to 5 days incubation at 30 ^{0} C.

2.2.6. Morphological, biochemical, Biolog and 16S rRNA gene based characterizations of the SBJ1357

Bacterial isolates was characterized using the morphological, biochemical and Biolog tests and technique.

2.2.6.1. Morphological characterization using Gram staining

The bacterial cultures were grown overnight and Gram-staining performed as according to the standard method described elsewhere (Appendix 3).

2.2.6.2. Biochemical characterization

2.2.6.2.1. Indole production

Autoclaved nutrient broth (Appendix 4) was inoculated and incubated at 30 ^{0}C for 24 to 48 h. Kovac's reagent (Appendix 5) was added to the broth and observed for the formation of red ring, which was used as index for positive test.

2.2.6.2.2. Methyl red

Autoclaved MR-VP broth (Appendix 6) was inoculated and incubated at 32 ^{0}C for 24 to 48 h. Methyl red (Appendix 7) was used as an indicator for the test and red colour produced was considered as positive test.

2.2.6.2.3. Voges-proskauer

Autoclaved MR-VP broth (Appendix 6) was inoculated and incubated at 32 ^{0}C for 24 to 48h. Barrit's reagent (Appendix 8) was added and observed for change in colour to red, which was used as an index for positive test.

2.2.6.2.4. Citrate utilization

Autoclaved Simmon's citrate agar slants (Appendix 9) were streaked with and inoculated at 32 ^{0}C for 24 to 48 h. Change in color from green to blue was considered as positive test.

2.2.6.2.5. Catalase

Autoclaved nutrient agar slants (Appendix 10) were inoculated with each test organism and incubated at 30 ^{0}C for 24 to 48 h. 3 % H_2O_2 was added to the slants and was observed for the appearance of bubbles of O_2.

2.2.6.2.6. Oxidase

Oxidase disc was moistened with distilled water. Cultures with the help of sterilized loop were spread on oxidase disc. The colour of disc changes to deep purple which showed a positive test while colorless spot indicate negative test.

2.2.6.2.7. Nitrate reduction

Autoclaved trypticase nitrate broth (Appendix 11) tubes were inoculated and incubated for 24 to 48 h at 30 ^{0}C. 5 drops of solution A was added followed by few drops of solution B to all nitrate broth cultures and observed for red color development.

2.2.6.3. Biolog based identification of SBJ1357

The strain SBJ1357 was grown on Biolog BUG® agar for identification by the Biolog® system (Biolog Inc., Hayward, CA, USA) following the standard method described by Nautiyal *et al.* (2007). For this, strain SBJ1357colony was picked and was plated as a lawn on to a BUG® agar plate. The strain SBJ1357 cells from BUG® agar plate was used to prepare cell suspension in saline water and inoculated into BIOLOG GN plate (150 µlwell^{-1}), which was then incubated at temperature 30 ^{0}C. The plate after 24 and 48 h of incubation periods was measured with a microtiter plate reader at absorbance of 595 nm. The Biolog Microlog® bacterial identification system consists of databases combined with specialized 96 well plates. A panel of 95 different substrates gives a very distinctive and repeatable pattern of purple wells for "Metabolic Fingerprint". The Gram-negative panel and database were used for SBJ1357 identification.

2.2.6.4. 16S rRNA gene based characterization

The 16S rRNA gene of the atrazine degrader strain SBJ1357 was cloned and sequenced. The detailed method is described in the section (2.2.8).

2.2.7. Assessment of the traits of the strain SBJ1357 responsible for the broad spectrum antimicrobial activity.

2.2.7.1. Qualitative assessment of siderophore production by SBJ1357

For the detection of siderophore, strain SBJ1357 was grown in minimal medium for 24 h on a rotary shaker at 30 °C. The Chrome Azurol S (CAS) agar assay described below was used to detect siderophores.

2.2.7.1.1. Chrome Azurol S (CAS) Agar

The CAS plates were used to check the culture supernatant for the presence of siderophore. The CAS assay is the universal chemical assay for the detection of siderophores. It is based on the high affinity of siderophores for ferric iron, where by ferric iron bound to dye, is complexed and released from the dye. The blue color of the medium is due to the dye complexed with iron. When siderophore is added, the siderophore binds the ferric iron, releasing the free dye, which is orange in color.

Fe3+-dye (blue) + siderophore ***Fe3+-siderophore + dye (orange)***

Hence, the presence of siderophore is indicated by a color change from blue to orange.

The CAS plates were prepared in three separate steps.

(1) Preparation of CAS indicator solution.

(2) Preparation of basal agar medium.

(3) Preparation of CAS agar plates.

2.2.7.1.2. Preparation of CAS indicator solution

Initially, 60.5 mg of chrome azurol S (CAS) was dissolved in 50 ml of ultra pure H_2O. 10 ml of Fe III solution (27 mg $FeCl_3 \cdot 6H_2O$ and 83.3 µL concentrated HCl in 100 ml H_2O) was added, along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml RO water. The HDTMA solution was added slowly while stirring, resulting in a dark blue solution (100 ml total volume), and the same was autoclaved.

2.2.7.1.3. Preparation of basal agar medium

In a 250 ml flask, 3 g 3-(N-morpholino) propane sulfonic acid (MOPS) (0.1 M), 0.05 g NaCl, 0.03 g KH_2PO_4, 0.01 g ammonium chloride (NH_4Cl), and 0.05 g L-asparagine was dissolved in 83 ml ultra pure water. The pH of the solution was adjusted to 6.8 using 6 M NaOH. The total volume was brought to 88 ml using RO water, and 1.5 g agar was added to the solution while stirring and heating until melted. The solution was then autoclaved.

2.2.7.1.4. Preparation of CAS agar plates

The autoclaved basal agar medium was cooled to 50 °C in a water bath. The CAS indicator solution was also cooled to 50 °C, along with a 50 % solution of glucose. Once cooled, 2 ml of the 50 % glucose solution was added to the basal agar medium with constant stirring, followed by 10 ml of the CAS indicator solution, which was added carefully and slowly along the walls of the flask with constant stirring, but at a speed so as not to generate any bubbles. Once mixed thoroughly, the resulting solution (100 ml) was poured into sterile plastic plates, each plate receiving approximately 25 ml of blue agar. Under minimal iron conditions, siderophore is produced and released into the culture medium. To detect the siderophore production strain SBJ1357 was grown in iron-restricted (0.5 µM added iron) modified minimal medium. After 24 h of growth, the culture was centrifuged and the cell supernatant was separated and collected by centrifugation for 2 min at 13,500 rpm. Supernatant was applied to CAS plates by using a cork borer to make a well on the plate. Culture supernatant was added to the well (60 µl), and the plate was incubated at 30 ^{0}C temperature to develop. The production of siderophore was confirmed by the presence of an orange halo.

2.2.7.2. Qualitative assessment of HCN production by SBJ1357

Production HCN was observed according to the method of Bakker and Schipper (1987). Briefly, King's-B medium (Appendix 12) amended with 4.4 g glycine was prepared and poured 25 ml in disposable Petri dish. After solidification the tested strain SBJ1357 was streaked. Then sterilized Whatman No.1 filter paper saturated with 1 % solution of picric acid and 2 % sodium carbonate was placed in the upper lid of a Petri dish. The Petri dish was then sealed with parafilm and incubated at 30 ^{0}C for 5 days .A change in color of the filter paper from yellow to reddish brown as an indication of cynogenic activity was noticed.

2.2.7.3. Assessment of PCA production by SBJ1357

2.2.7.3.1. Qualitative estimation of PCA

The tested strain was grown at 28 ^{0}C in a 100 ml conical flask containing modified medium that favored PCA production. The supernatant of 3 days cultures was

acidified to pH 2.0 with concentrated hydrochloric acid and extracted with equal volumes of benzene. The organic phase was pooled and dried by evaporation. The dry residue was dissolved in 0.1M NaOH and absorbance was read at 367 nm.

2.2.7.3.2. Cross-feeding assay for detection of acylated homoserine lactones (AHLs) produced by SBJ1357

Agrobacterium tumefaciens A136 (Ti⁻)(pCF218) (pCF372) (Fuqua and Winans, 1996) was used as an indicator strain for the detection of AHLs production by SBJ1357. The genetic element pCF218 codes for the TraR protein, an AHL-responsive transcription factor that recognizes N-3-(oxooctanoyl)-L-homoserine lactone as well as a wide range of related AHLs. A TraR-regulated *tra*I-*lac*Z reporter is carried on pCF372. The indicator strain was maintained on LB agar (Appendix 12) supplemented with spectinomycin and tetracycline. LB agar plate contained the 40 µl of X-Gal (5-bromo-4-chloro-3-indolyl--D-galactopyranoside) (20 mg/ml stock solution in DMSO) was used for the cross-feeding assays. These assays consisted of streaking the AHL reporter strain, *A. tumefaciens* A136 (pCF218) (pCF372), on the plate and then streaking the SBJ1357 culture to be tested approximately 1cm distant. If AHLs are produced by SBJ1357, they will diffuse through the agar and as a result activate *tra*I-*lac*Z fusion in the reporter strain (Fuqua and Winans, 1996).

2.2.8. Nucleotide sequencing of the 16S rRNA, *hcn* and *phz* genes of the SBJ1357

2.2.8.1. Isolation of DNA from SBJ1357

Genomic DNA from SBJ1357 was isolated and purified by a cetyltrimethylammonium bromide (CTAB) miniprep procedure (Ausubel *et al.*, 1995). Briefly, cells were homogenized with 0.5ml CTAB buffer (2% CTAB; 100 mM Tris-HCl pH 8.0; 20 mM EDTA ; 1.4 M NaCl; 1 % SDS) containing 10 mM β-mercaptoethanol and incubated at 60 ^{0}C for 20 min. The homogenate was mixed with phenol: chloroform: iso-amyl alcohol (25:24:1) and centrifuged at 14,000 rpm for 6 min. The aqueous phase was precipitated with an equal volume of isopropanol and 1/10th volume of 3M sodium acetate (pH 5.2) and kept overnight at -20 ^{0}C. The pelleting of DNA was carried out at 14,000 rpm (4 ^{0}C) for 15 min.

The pellet was washed with 70 % ethanol; vacuum dried and dissolved in 50 µl nuclease-free water. The concentration was determined and the preparation was stored in ethanol at -80 °C.

2.2.8.2. Polymerase chain reaction ((PCR) based amplification of SBJ1357 genes

The PCR of selected SBJ1357 genes was carried out using the following protocol.

DNA	2.0µl
10 X PCR buffer	2.5µl
$MgCl_2$ (25 mM)	1.5µl
dNTPs (2 mM)	1.0µl
Forward primer (25 pmol)	1.0µl
Reverse primer (25 pmol)	1.0µl
Taq DNA-Polymerase (3 u/µl)	1.0µl
H_2O	15.0µl
Total	**25.0µl**

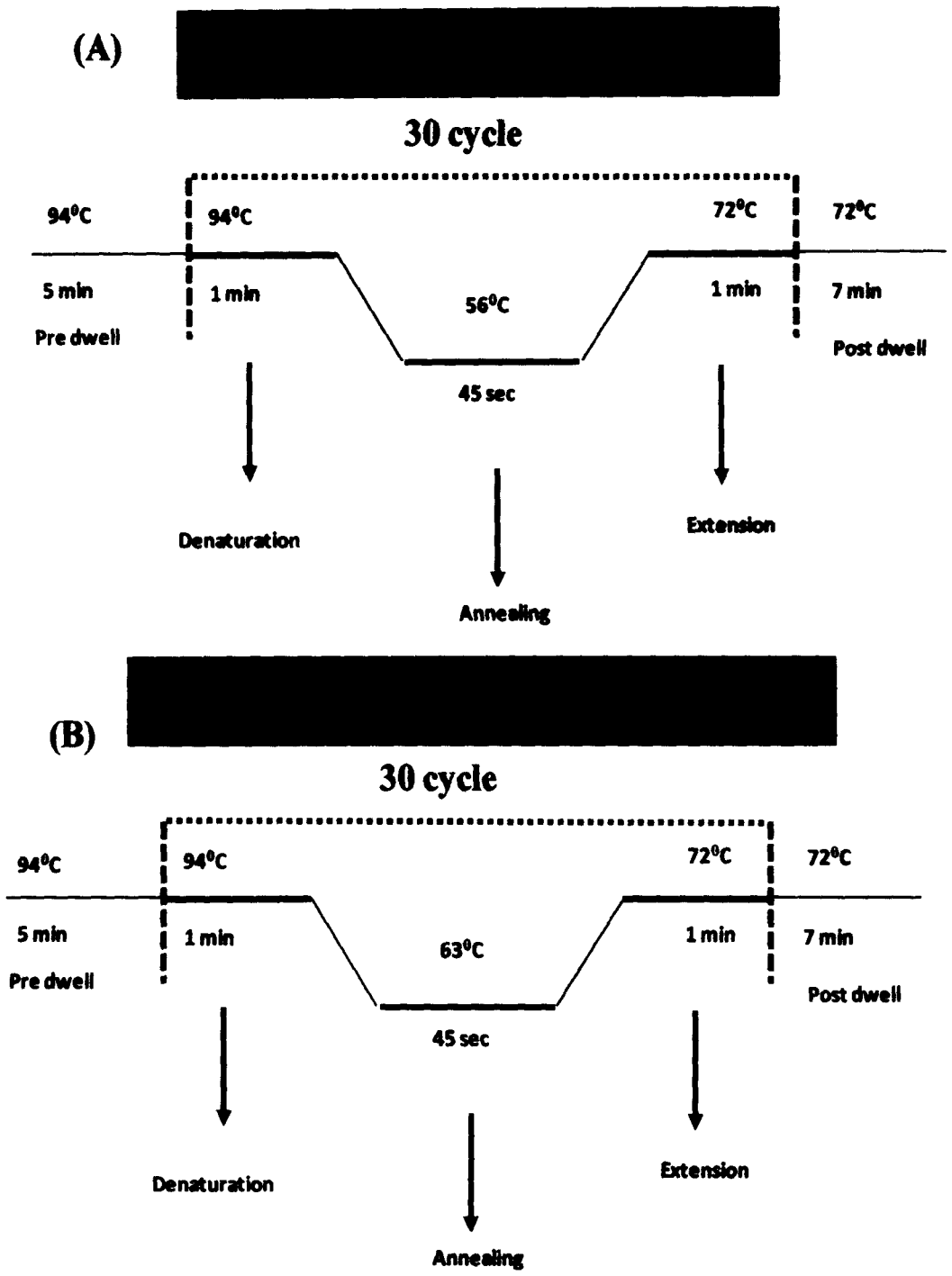


Fig.3. PCR conditions of the targeted genes of the strain SBJ1357.

2.2.8.3. Agarose-gel electrophoresis of PCR amplified SBJ 1357 genes

PCR amplicons were separated using 0.8 to 2.0 % (w/v) agarose gels in TAE buffer containing ethidium bromide (0.2 μgml^{-1}) with 4 V/cm and examined by UV light at 254 nm, using a transilluminator. Gels were documented, analyzed and photographed to record results.

2.2.8.4. Extraction of PCR amplicons from agarose gel.

PCR amplicons were recovered from agarose gel using QIAEX II agarose gel extraction Kit. This protocol was designed for the extraction of 40 bp to 50 kb DNA fragments from 0.3-2 % standard agarose gels in TAE buffer. DNA molecules were adsorbed to QIAEX II silica particles in presence of high salts. All non-nucleic acid impurities such as agarose, proteins, salts, and ethidium bromide were removed during washing steps. The desired amplicons were excised from the agarose gel under UV light. The gel slice was weighed and added with 3 volumes of Buffer QG to 1 volume of gel and added the 10 µl of QIAEX II buffer. The mixture was incubated at 50 °C for 10 min to solubilize the agarose and binding of DNA. The mixture was vortexed every 2 min and centrifuged for 30 sec. The supernatant was carefully removed and pellet thus obtained was washed with 500 µl QG Buffer and then twice with 750 µl PE Buffer. The pellet containing DNA was air-dried and dissolved in 10 mM Tris-HCl. The preparation was incubated at 50 °C for 5 min and centrifuged for 30 sec. The supernatant was carefully pipetted out in a clean microfuge tube.

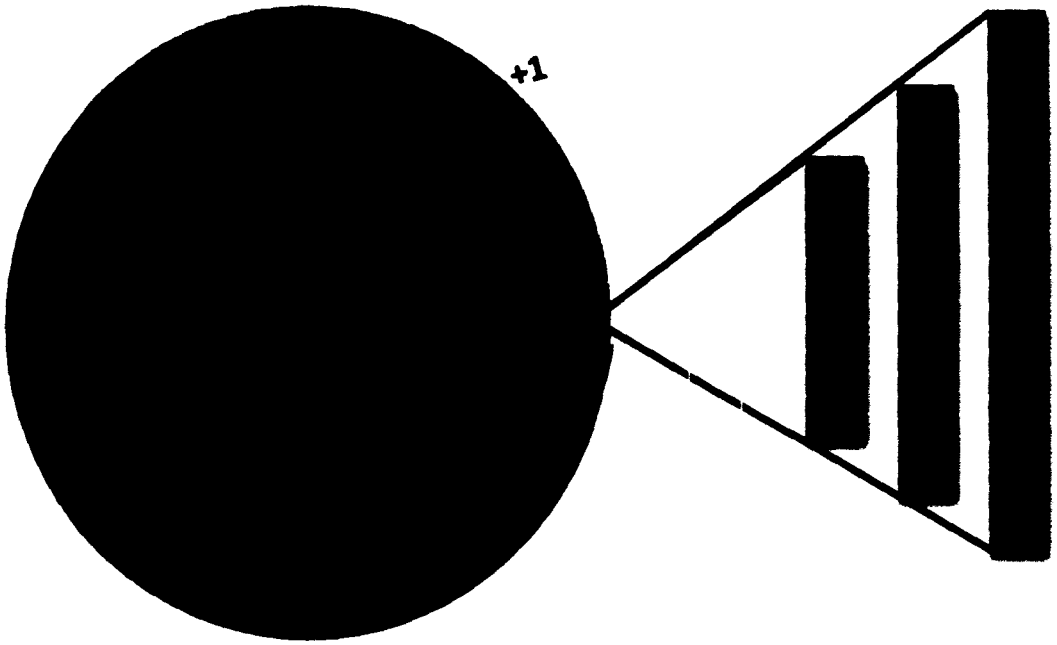


Fig.4. Genetic map of the pDrive plasmid cloning vector and site of strain SBJ1357 genes cloned in this study.

2.2.8.5. Ligation of the strain SBJ1357 genes in plasmid cloning vector

Ligation reaction was performed using vector: amplicon in the ratio of 1:3 as per following procedure. The ligation mixture was centrifuged and incubated overnight at 4^0C.

Recipe for ligation mix:

Reagents	Volume added (µl)
Vector	1.0
2 X ligation buffer	5.0
Amplicon	3.0
Nuclease free water	1.0
Total	**10.0 µl**

2.2.8.6. Preparation of competent cells ($CaCl_2$ method) of host strains

The single colony of host strain was inoculated in 5 ml LB medium and incubated at 37 °C with continuous shaking. After 16 h, 1ml of the pre-culture was inoculated in 100 ml fresh medium and incubated at 37 °C until OD of culture reaches 0.3 at 600 nm. The culture was kept on ice for at least 15 min at 4 °C in pre-cooled sterile tubes. The cells were harvested and centrifuged at 5,000 rpm for 5 min and the supernatant was discarded. The pellet was re-suspended thoroughly in a small volume of ice-cold 100 mM $CaCl_2$. The cell suspension was diluted with the $CaCl_2$ solution to a final volume of 30 to 40 ml and left on ice for 25 min with occasional shaking. Further, the cells were spin down and the pellet was re-suspended in 5 ml $CaCl_2$ containing 15% glycerol. The suspension was dispensed in 100 to 400 µl aliquots and stored at -80 °C. The transformation efficiency of the bacterial cells was evaluated.

2.2.8.7. Transformation of recombinant plasmid cloning vector into competent cells of host strain

The culture of competent bacterial cells was thawed and 20 ng ligated vector (Fig.4) was added to 100 µl competent cells in cold 1.5 ml microfuge tube, mixed carefully and kept on ice for 20 min. The mixture was then heat-shocked at 42 °C

for 90 sec, 1 ml antibiotic free LB medium was added and the tube was incubated at 37 °C for 30 min.

2.2.8.8. Selection of transformants

The transformants were selected on the basis of blue/white colony color. The white colonies were selected and subsequently plated on LBA plate containing ampicillin, X-gal and IPTG as described earlier. The plate having individual transformant in grid served as master plate and store at 4°C.

2.2.8.9. Plasmid DNA isolation from clones

Clones from master plates were grown overnight in presence of ampicillin/kanamycin (100 μgml^{-1}) for the purification of recombinant plasmid DNA using the boiling lysis (Sambrook *et al.*, 1989) and miniprep methods (Birnboim and Doly, 1979)

2.2.8.9.1. Boiling lysis method of plasmid isolation

Bacterial cells from the overnight culture were centrifuged for 5 min at 8,000 rpm and supernatant was removed by gentle aspiration. The pellets were re-suspended in solution A (100 µl) containing 4 µl lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0) and boiled for 1 min. The bacterial lysate was centrifuged at 12,000 rpm, for 10 min at room temperature and the pellet was removed. The plasmid DNA from the remaining supernatant was precipitated with equal volume of ice-cold iso-propanol. The pellet was recovered by centrifugation 12,000 rpm for 15 min at 4 °C. The pellet was washed twice with 70 % ethanol, vacuum dried and finally dissolved in 50 µl sterile MQ water.

2.2.8.9.2. Mini-preparation method of plasmid isolation

Solution A	25mM	HCl
	50mM	Glucose
	10mM	EDTA
		pH 8.0
Solution B	200mM	NaOH
	1% w/v	SDS
Solution C	3M	Na-Acetate

Cells from the overnight freshly grown bacterial culture (~1.5 ml) were harvested by centrifugation at 12,000 rpm for 5 min. The pellet was subsequently re-suspended in solution A (100 µl) and incubated for 5 min at room temperature. Thereafter, solution B (200 µl) and solution C (300 µl) were added. The mixture was incubated for at least 15 min on ice and centrifuged for 10 min at 12,000 rpm at room temperature. The supernatant was transferred to a fresh tube and plasmid was precipitated from the supernatant by adding two volume of ethanol. The pellet was recovered by centrifugation at 14,000 rpm for 15 min, vacuum dried and finally dissolved in 50 µl sterile MQ water.

2.2.8.10. Confirmation of transformants

The presence of gene insert in transformants was confirmed by restriction analysis. Isolated plasmid DNA was digested with restriction enzyme *Eco* RI in the 20 µl of total reaction mixture containing (10X reaction buffer, 2 µl; Plasmid DNA, 4 µl; *Eco* RI, 1µl (10 U/µl), and MQ water, 13 µl) at 37 ^{0}C for 4 h. The digested sample was electrophoresed on 1 % agarose gel and the insert size was ascertained in comparison with PCR amplicons. All clones were tested for the presence of the genes by PCR using respective primers.

2.2.8.11. DNA sequencing of cloned genes

The cloned genes were sequenced using an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems) at DNA Sequencing Facility, Department of Biochemistry, South Campus, University of Delhi, India. The sequencing reactions were carried out with AmpliTaq^{TM} DNA Polymerase FS dye terminator cycle sequencing chemistry using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Applied Biosystems) according to the manufacturer's protocol. Reaction mixes were subjected to 25 cycles in an Applied Biosystems GeneAmp 9700 Thermal cycler.

2.2.8.12. Multiple sequence alignments, phylogeny determination and bioinformatic analysis of strain SBJ 1357 genes and their proteins

The sequences were compared with other sequences retrieved from GenBank using the program Blast (Altschul *et al.*, 1997). The homologous sequences were aligned

using ClustalW with default parameters (Thompson *et al.*, 1994). The alignment was corrected by in coding regions to maintain the alignment of the encoded amino acids. Phylogenetic relationships of the SBJ1357 on the basis of RNA and protein coding genes were determined and trees were constructed by neighbour-joining (NJ) method (Saitou and Nei, 1987). The nucleotide pair-wise genetic distances were corrected by using Kimura two-parameter methods (Kimura, 1980), including transitions and transversions unweighted and handling gaps by pairwise deletion. The significance of the internal branches was evaluated by using 1000 bootstrap replications. All branches with <40 % bootstrap support were judged as inconclusive and were collapsed. Branch lengths for all trees were normalized to 0.02 % divergence.

2.2.9. Assessment of the plant growth promoting activities

2.2.9.1. Quantitative assessment of indole acetic acid (IAA) production by strain SBJ1357

The amount of IAA produced by the strain SBJ1357 was determined according to the method of Bric *et al.* (1991). Briefly, an aliquot of 2 ml supernatant obtained from bacterial culture grown in LB broth medium (Appendix 13) without and with tryptophan (250 and 500 $\mu g ml^{-1}$) was mixed with 100 µl of 10 mM orthophosphoric acid and 4 ml of reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$). The absorbance of pink color developed after 25 min incubation was read at 530 nm. The IAA concentration in culture was determined using a calibration curve of pure IAA as a standard following the linear regression analysis.

2.2.9.2. Qualitative and Quantitative assessment of phosphate solubilizing activity of strain SBJ1357

The phosphate-solubilizing activity of the strain SBJ1357 was determined by measuring the zone size formed by solubilization of insoluble phosphate on Pikovskaya's agar plates (Appendix 14). Furthermore, the soluble phosphate released was quantitated following the method of King (1932). In brief, 100 ml of Pikovskaya's liquid medium was inoculated with 0.5 ml of culture of strain SBJ1357. At different time intervals during growth up to 5 days, 10 ml of culture

was removed and centrifuged at 10,000 rpm for 30 min. Aliquot of 0.4 ml of supernatant was mixed with 1 ml of chloromolybdic acid and 150 µl chlorostannous acid, and the volume adjusted to 5 ml with distilled water. The absorbance of blue colour developed was read at 600 nm and the amount of phosphate released was determined using the calibration curve of KH_2PO_4.

Chapter-III

Results

The present study deals with the isolation and characterization of atrazine degrader bacterial strain (SBJ1357) from wheat rhizosphere soil. The rhizosphere samples were processed for screening of atrazine degrading bacteria and finally the strain SBJ1357 has been chosen out of 50 strains as the most promising. The strain SBJ1357 also has been exhibited the broad spectrum antimicrobial activity. Beside, this strain also having the intrinsic ability of IAA production and inorganic phosphate solubilization for plant growth promotion.

3.1. Soil characteristics

For optimum activity of any PGPR, it is essential to know the soil characteristics. With this point of view the major factors like soil pH, temperature and water holding capacity (WHC) have been determined. The data revealed the WHC as 50 %, temperature, 18 ^{0}C and pH of the soil as 8.0.

3.2. Assessment of its biodegrading capability of SBJ1357

Bacterial strain with the inherent ability of degrading atrazine was obtained from sandy loam soil with a history of pesticide application. Out of 50 isolates, the SBJ1357 was specifically chosen based on its relatively higher growth efficiency and enhanced tolerance up to 500 μg ml^{-1} atrazine in mineral salt medium (Fig.5). The strain showed exponential growth upto 16 h before transition to the stationary phase. The maximum growth rate (μ_{max}) for strain SBJ1357 noticed was 9.6 logCFU h^{-1}. The MS medium supplemented with 500 μg ml^{-1} atrazine, suggests the ability of the strain to utilize atrazine as the sole source of carbon. (Fig.6) shows the kinetics of atrazine degradation in soil microcosm. The data exhibit almost >90% transformation of atrazine within 20 days (Figs.7 and 8). The degradation isotherm demonstrates the time-dependent disappearance of atrazine with a rate constant as 0.173 day^{-1} following the first-order rate kinetics. However, the parallel control, comprising sterilized soil containing an identical amount of atrazine, did not exhibit any atrazine degradation.

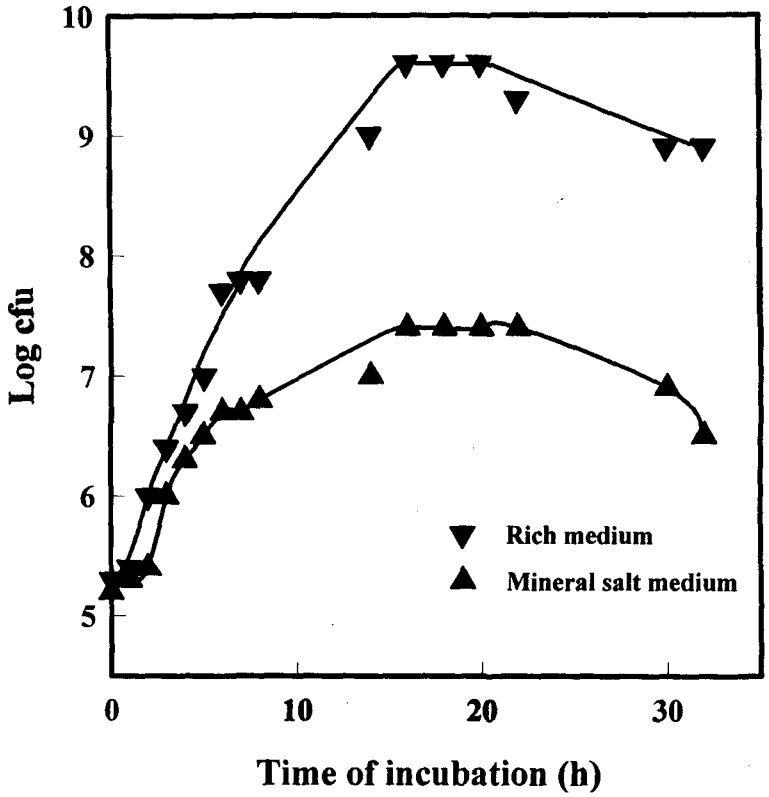


Fig.5. Growth kinetics of strain SBJ1357 in minimal salt medium salt medium amended with atrazine (500 μM) as a sole source of carbon.

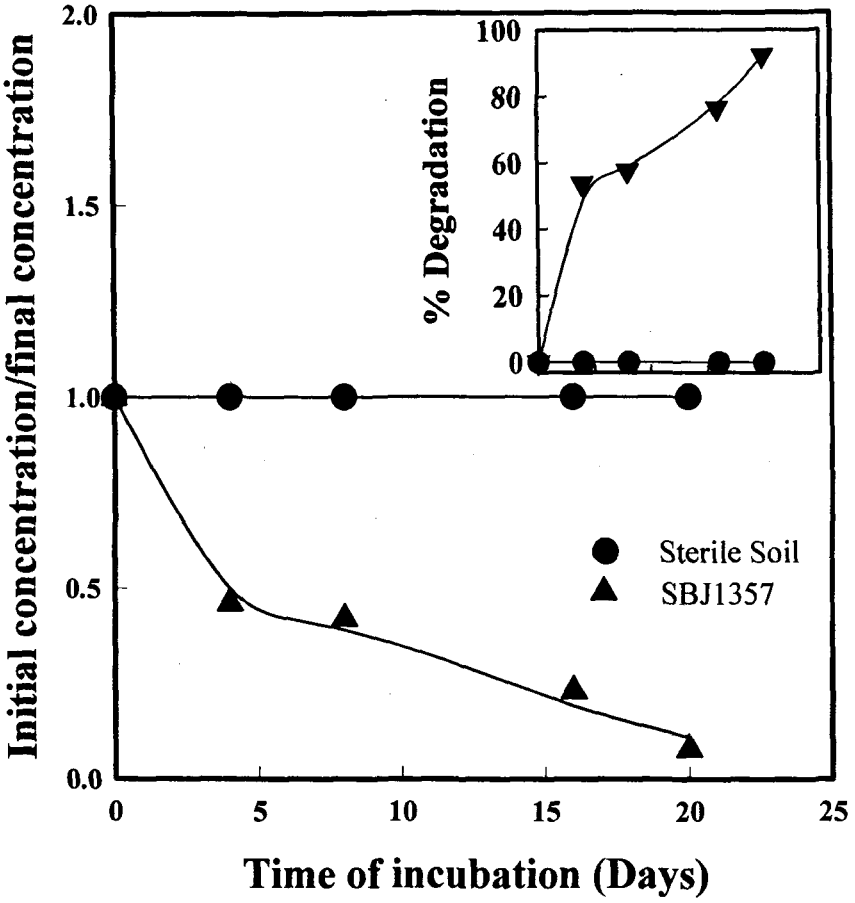


Fig.6. Rate of disappearance of atrazine in soil microcosm augmented with pure culture of atrazine degrading strain SBJ1357. The amount of atrazine remaining in amended soil at different time points was estimated by HPLC analysis. The inset shows the percent degradation of atrazine as a function of time.

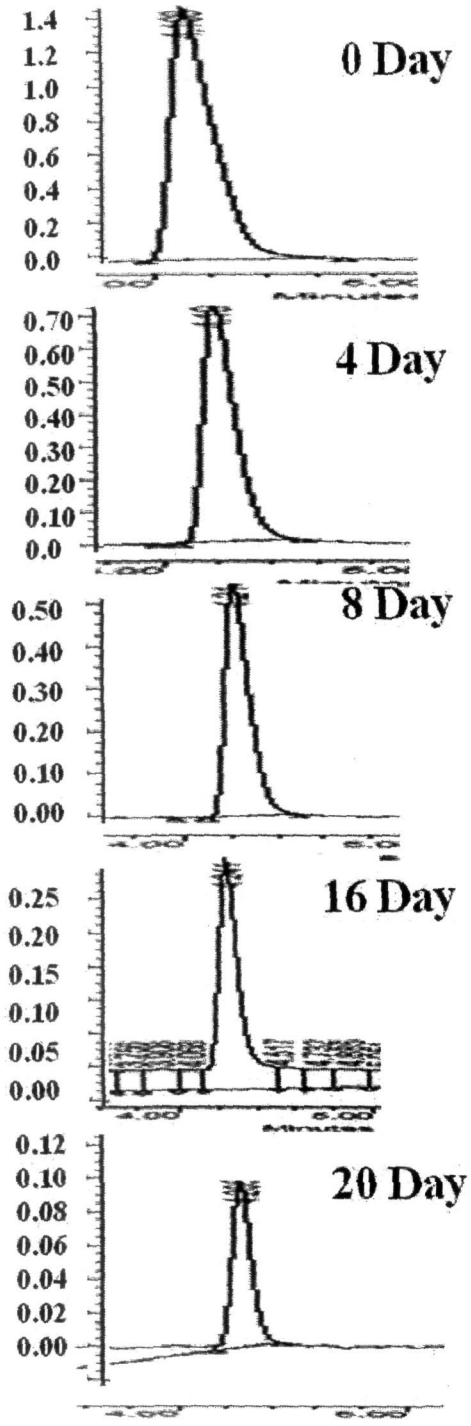


Fig.7. HPLC chromatogram showing the disappearance of atrazine and metabolites in soil microcosm augmented with pure culture of atrazine degrading strain SBJ1357. The amount of atrazine remaining in amended soil at different time points was estimated by HPLC analysis (0, 4, 8, 12, 16 and 20 days).

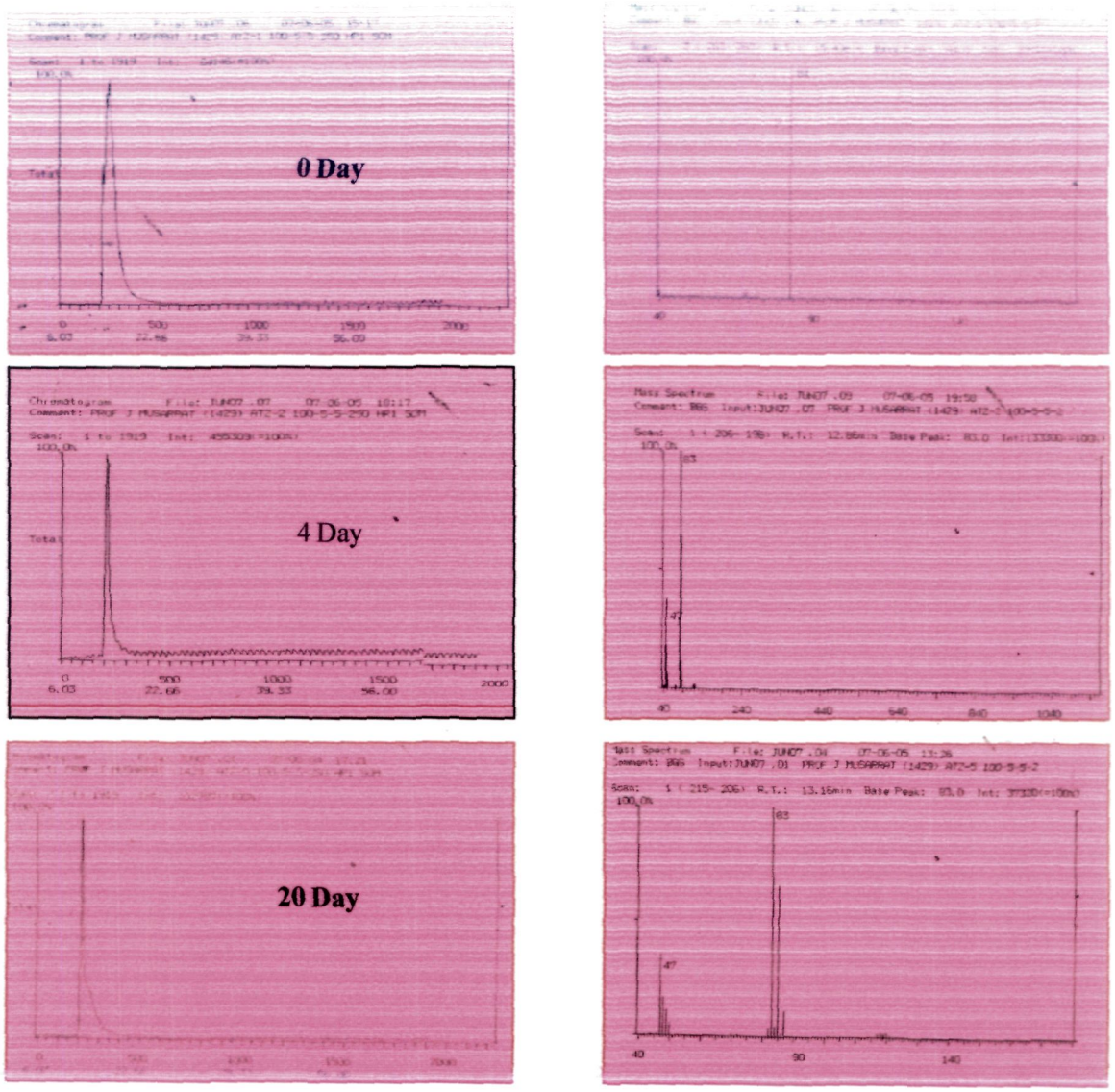


Fig.8. GC-MS shows the atrazine metabolites in soil microcosm augmented with pure culture of atrazine degrading strain SBJ1357. The types of atrazine metabolites in amended soil at different time points was evaluated by GC-MS analysis (0, 4, and 20 days).

3. Biochemical tests, Biolog assay and 16S rRNA gene based characterization of the strain SBJ1357

The strain SBJ1357 was presumptively found to be *Pseudomonas* sp. based on the biochemical characteristics (Fig.9 and Table 1) and Biolog (Fig.10A, B and C). Considering the importance of valuable activities of strain SBJ1357, its definitive characterization was carried out on the basis of 16S rRNA gene sequence. For this purpose genomic DNA from the strain has been isolated and purified. The purity of DNA was checked both by measuring the optical density at 260 and 280 nm as well as on 0.8% agarose gel. The $A_{260/280}$ ratio of purified DNA in the range of 1.85 to 1.92 and appearance of the single band on gel confirmed the purity of the isolated genomic DNA (Fig.11). The purified genomic DNA was used for the PCR amplification of full length16S rRNA gene of strain SBJ1357. The PCR product run on 2% agarose gel and presence of the single band of approximately 1.5 kb suggested the successful amplification of the complete 16S rRNA gene (Fig.12A). The 16S rRNA gene has been cloned and sequenced (Fig.12B). The BLASTn was performed using the sequence of 16S rRNA gene of the test strain, which showed the highest sequence homolog of SBJ1357 with the *Pseudomonas aeruginosa* strains (Fig.13). Further more, phylogenetic tree was constructed with neighboring joining (NJ) method (Figs.14 and 15). Thus, the molecular characterization of strain SBJ1357 based on 16S rRNA gene validates the strain SBJ1357 as *Pseudomonas aeruginosa.*

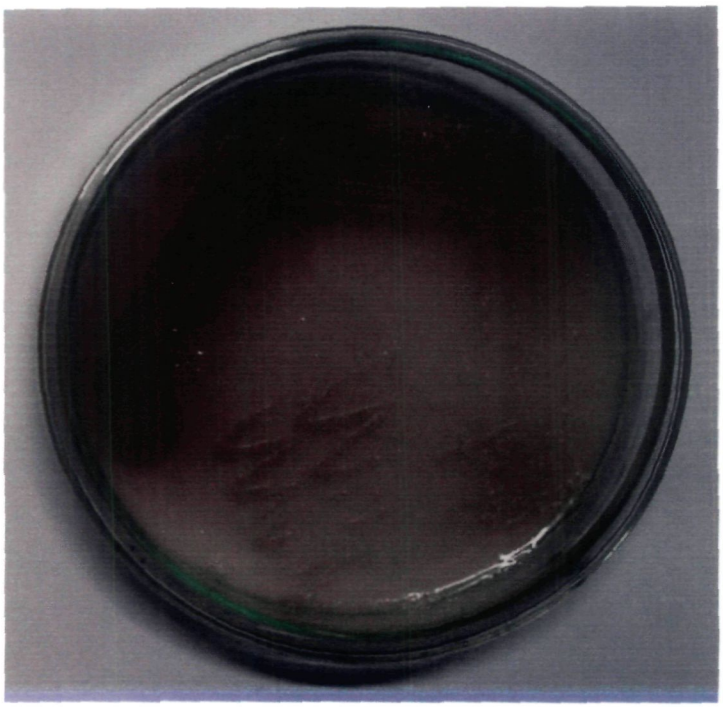
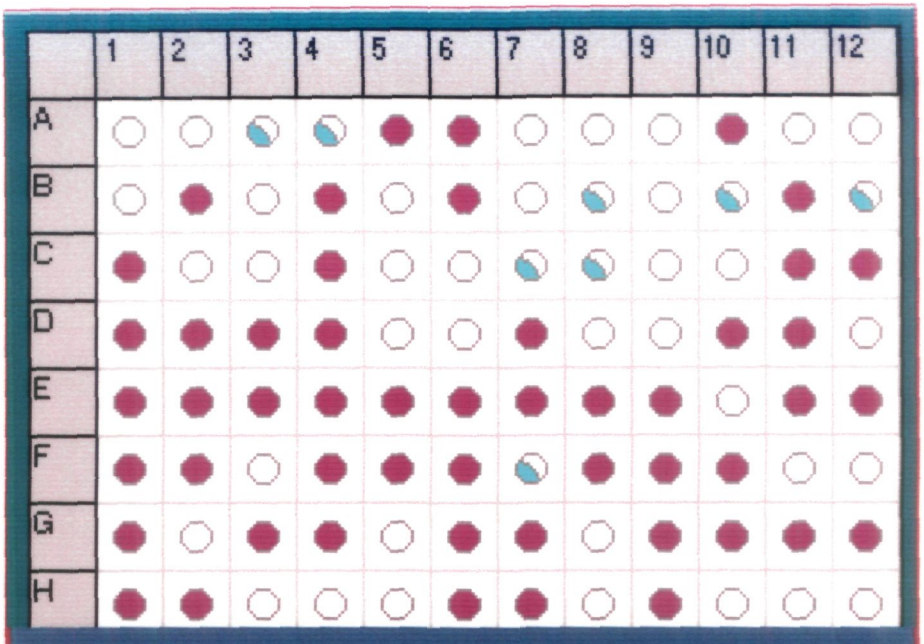


Fig. 9. Colony characteristics of SBJ1357 on LB agar medium

Table.1. Morphological and biochemical characteristics of atrazine degrading soil bacterial strain SBJ1357.

Morphological characteristics	**Remark**
Colony Morphology	Spherical, greenish
Gram stain	-ve
Biochemical characteristics	
Indole test	–
Methyl red	–
Voges proskauer	–
Citrate utilization	–
Fructose fermentation	+
Glucose fermentation	+
Sucrose fermentation	–
Mannitol	–
Catalase	+
Oxidase	+
Nitrate reduction	–
Starch hydrolysis	–
Gelatin hydrolysis	–
Presumptive identification	***Pseudomonas* Sp.**

(A)



(B)

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	b	b	+	+	-	-	-	+	-	-
B	-	+	-	+	-	+	-	b	-	b	+	b
C	+	-	-	+	-	-	b	b	-	-	+	+
D	+	+	+	+	-	-	+	-	-	+	+	-
E	+	+	+	+	+	+	+	+	+	-	+	+
F	+	+	-	+	+	+	b	+	+	+	-	-
G	+	-	+	+	-	+	+	-	+	+	+	+
H	+	+	-	-	-	+	+	-	+	-	-	-

(C)

Species ID: Pseudomonas aeruginosa

ML4	NAME	PROB	SIM	DIST
=>1	Pseudomonas aeruginosa	99%	0.559	6.92
2	Pseudomonas citronellolis	0%	0.000	9.22
3	Pseudomonas pseudoalcaligenes ss pseudoalcaligenes	0%	0.000	11.65
4	Acinetobacter calcoaceticus/genospecies 3	0%	0.000	12.00
5	Pseudomonas lundensis	0%	0.000	12.73
6	Pseudomonas fulva	0%	0.000	12.73
7	Pseudomonas nitroreducens/azelaica	0%	0.000	12.93
8	Acinetobacter calcoaceticus/genospecies 1	0%	0.000	13.15
9	Pseudomonas agarici	0%	0.000	13.34
10	Pseudomonas putida biotype B	0%	0.000	13.35

Plate type - GN2
Strain - SBJ1357
Incubation time - 24h
Analysis software - MicroLog

Figs.10. Taxonomic characterization of the strain SBJ1357 using Biolog method.

(A) Pattern of different substrate as carbon source utilization by strain SBJ1357.

(B) The carbon substrate utilization pattern assessed by the micro plate reader.

(C) MicroLog software based taxonomic analysis of strain SBJ1357.

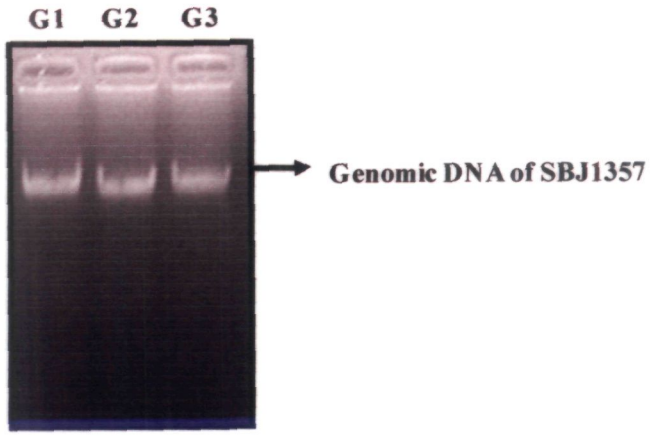


Fig.11. Lanes, G1 to G3; shows distinct bands of genomic DNA of SBJ1357 on 0.8% agarose gel.

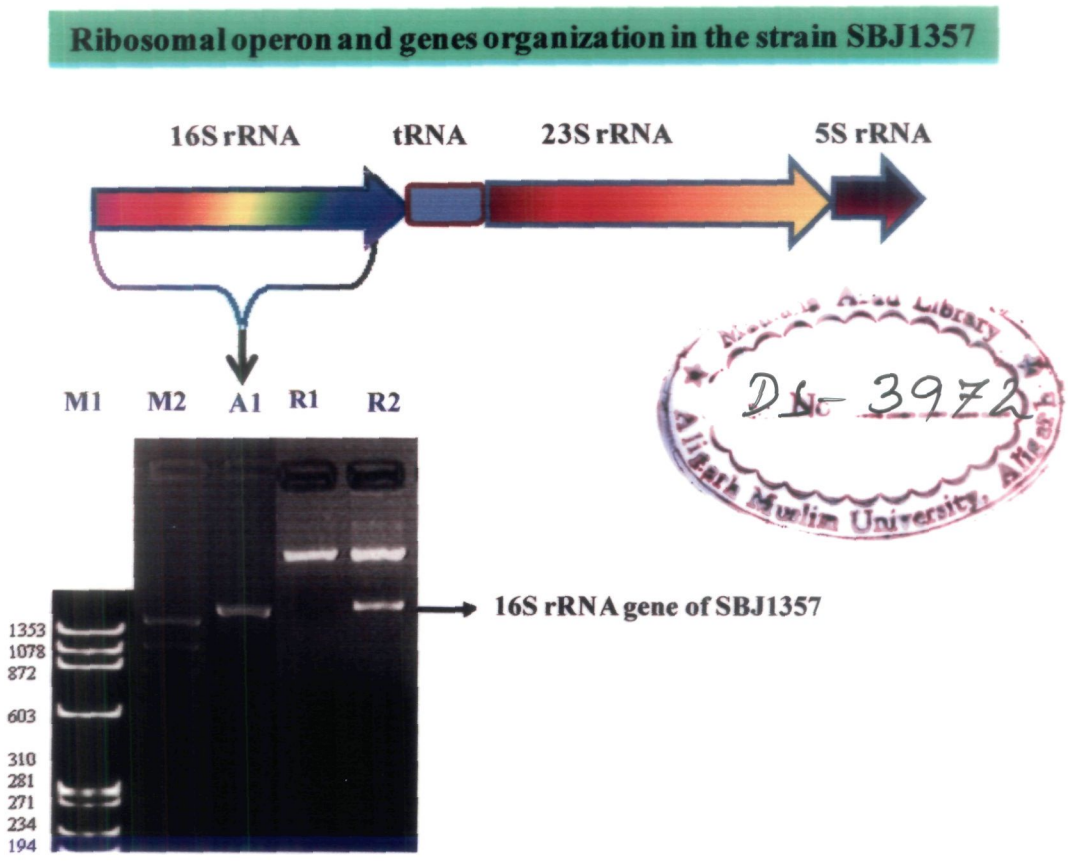


Fig.12. (A); Ribosomal operon organization of the SBJ1357 and targeted genes in this study; **(B)** Agarose gel electrophoresis of the amplified 16S rRNA gene from SBJ1357 genomic DNA

M1 and M2- □X174 *Hae* IIIDNA molecular weight marker.

A1- PCR amplified 16S rRNA gene (1.5 kb) of the SBJ1357 strain.

R1- *Eco*RI digested plasmid vector without 16S rRNA gene insert.

R2- *Eco*RI digested recombinant plasmid vector containing 16S rRNA gene insert.

Fig.13. 16S rRNA gene nucleotide sequences of strain SBJ1357.

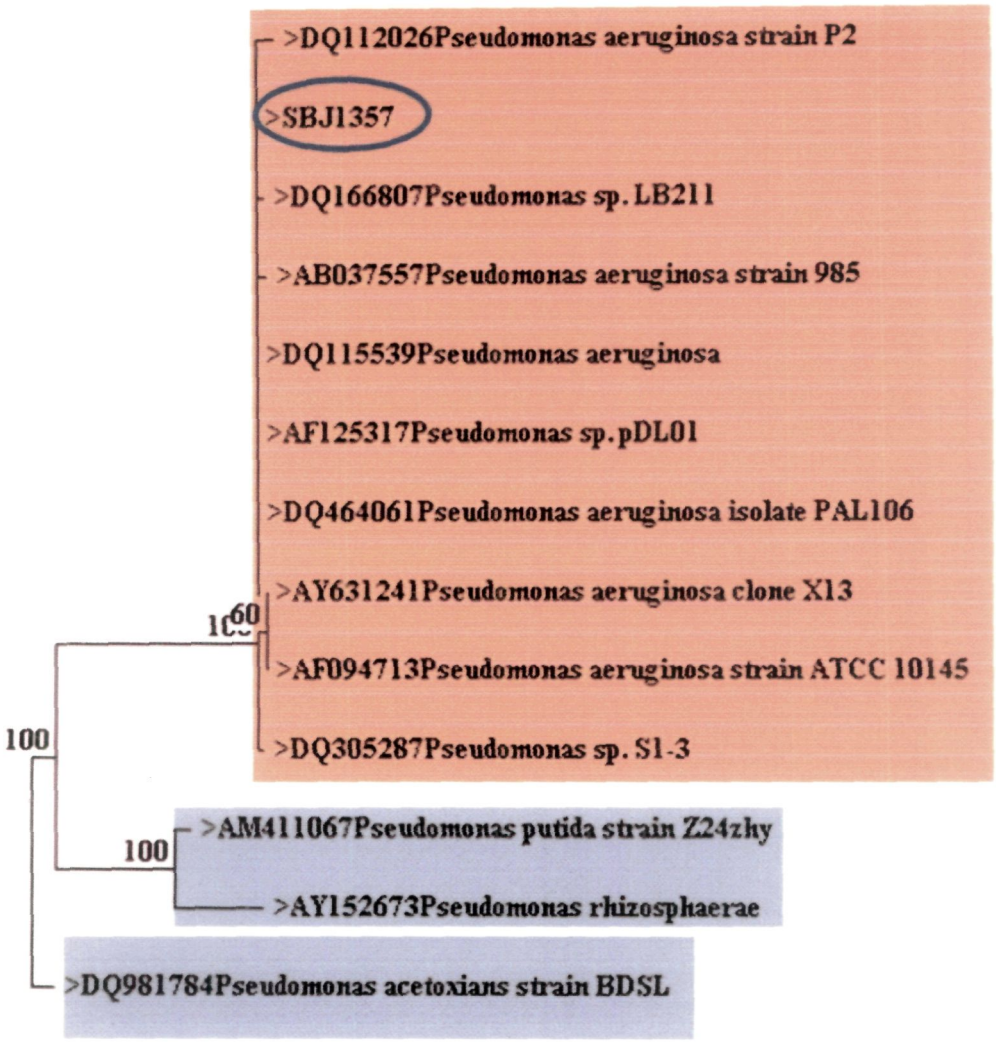


Fig.14. Phylogenetic relationship based on 16S rRNA gene nucleotide sequences of SBJ1357 with reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40% or greater are indicated on the tree.

Taxonomy Report

Bacteria	220 hits	34 orgs	[root; cellular organisms]
. environmental samples	34 hits	3 orgs	
. . unidentified bacterium	1 hits	1 orgs	
. . uncultured bacterium	31 hits	1 orgs	
. . bacterium enrichment culture clone CH7	2 hits	1 orgs	
. Proteobacteria	184 hits	30 orgs	
. . Pseudomonas	182 hits	29 orgs	[Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae]
. . . Pseudomonas aeruginosa group	132 hits	6 orgs	
. . . . Pseudomonas aeruginosa	122 hits	4 orgs	
. . . . . Pseudomonas aeruginosa PA7	8 hits	1 orgs	
. . . . . Pseudomonas aeruginosa UCBPP-PA14	8 hits	1 orgs	
. . . . . Pseudomonas aeruginosa PAO1	8 hits	1 orgs	

Fig.15. Illustrating the 16S rRNA gene based taxonomic report of the strain SBJ1357. The 16S rRNA gene based taxonomic report validated the taxonomic report of Biolog.

3.3. Broad spectrum antimicrobial activity of strain SBJ1357

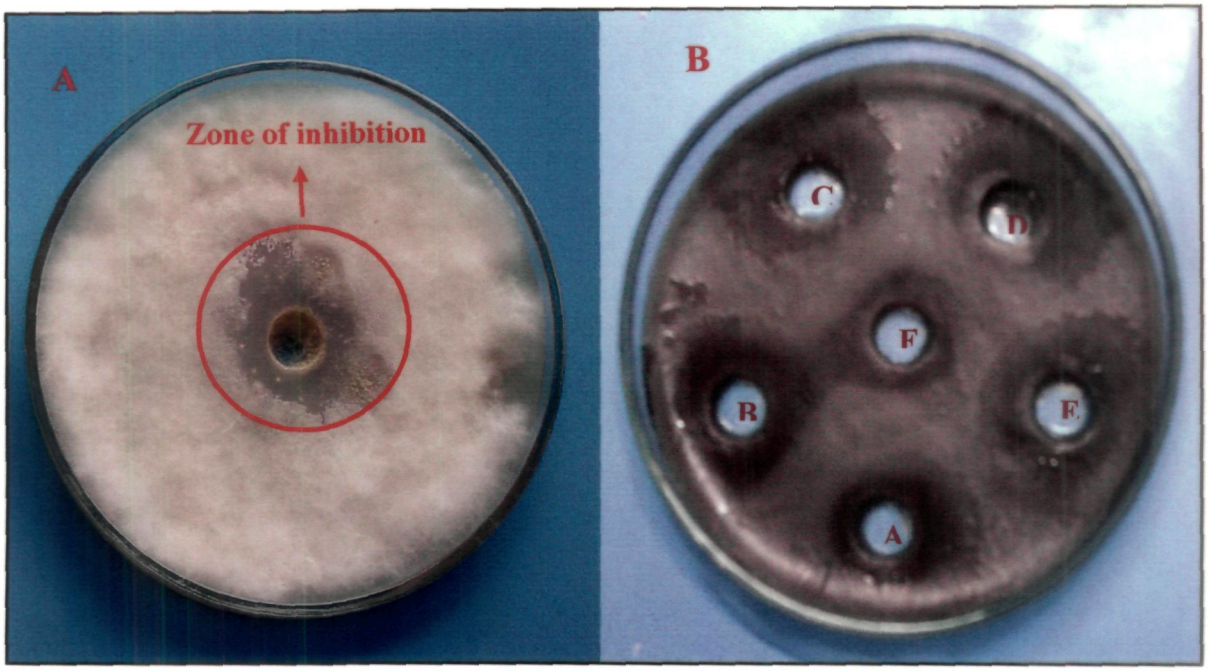
The strain SBJ1357 exhibited inherent broad spectrum antimicrobial activity against phytopathogen (*Fusarium oxysporum*) and human pathogen (*Candida albicans*) in antagonistic bioassay (Fig.16A and B). The pathogens species used in this study are common pathogen of crops (vegetable and legume) and humans. The formation of variably sized zones of inhibition suggests deferential sensitivity of the pathogens.

3.3.1. Assessment of inherent traits of SBJ1357 responsible for its broad spectrum antimicrobial activity.

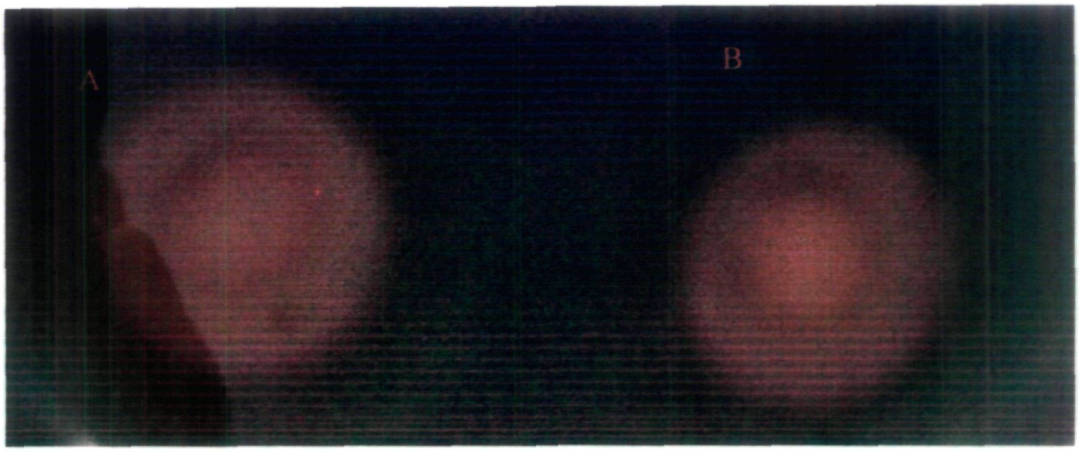
Inherent traits responsible for the broad spectrum antimicrobial activity of the strain SBJ1357 were assessed using the complete genome sequenced *Pseudomonas aeruginosa* strains (PAO1 and UCBPP-PA14) database. Production of siderophore, hydrogen cyanide (HCN) and phenazine-1-carboxylic acid (PCA) was assessed.

3.3.1.1. Siderophore production

The production of siderophore by strain SBJ1357 was validated by absorption spectral analysis, ferric chloride test and CAS assay. Appearance of a reddish-brown zone on CAS plates suggests the positive result for siderophore (Fig.17A and B). Eventually the siderophore produced by strain SBJ1357 has been identified as hydroxamate-type, on the basis of the ferric-siderophore complex with absorption maximum between 420-450 nm, which is the characteristic of hydroxamate-type siderophores.



Figs.16. (A) and (B); Broad spectrum antimicrobial activity of strain SBJ1357 against phytopathogenic fungi, *Fusarium oxysporum* and human pathogen yeast, *Candida albicans,* respectiveley.



Figs.17. (A) and (B); Chrome Azurol S (CAS) assay shows the siderophore producing ability of the strains SBJ1357 and NJ101 (as positive control, respectively.

3.3.1.2. Assessment of HCN production by SBJ1357and *In-Silico* analysis of *hcn*B&C genes.

Screening of the strain SBJ1357 for HCN production revealed a remarkable change in color from yellow to brown against the control, suggestive of HCN production. The effect of iron on HCN production was also evaluated. The level of HCN production under low iron condition decreased as compared to higher HCN production in the medium fortified with iron ($FeCl_3$, 75 $\mu g ml^{-1}$) (Fig.18A, B and C).

Although, the relationship to exist between the ability of Pseudomonads to produce HCN and their biocontrol capacity but information of comparative genomics and gene structure relatioship is very limited. In this study primer ACa and ACb were used for amplification of *hcnB&C* genes (Fig.19A). In tested strains (SBJ 1357), the *hcnB&C* fragment amplified 587 bp fragment (Fig.19B), it includes 156 bp of 1,404 bp of *hcn*B and 436 bp of 1,251 bp of *hcnC*. A single product of desired 587 bp in length was therefore obtained.

The PCR amplified partial *hcn*B&C genes of SBJ1357 strain were sequenced. The amplicon was successfully cloned in plasmid cloning vector. The presence of the desired gene insert in recombinant clones was validated through PCR and restriction analysis. The restriction digestion of clones with the inserts *hcn*B&C genes suggested the absence of internal *Eco*R I sites (Fig.19B). The randomly selected positive clone was sequenced, and BLAST analysis performed. The nucleotide and amino acid sequences of strain SBJ1357 showed the highest sequence homology with the other *Pseudomonas* spp. strains (Table 2). The sequenced *hcn*B&C genes were translated into amino acid sequences, which were found to be of 146 and 435 nucleotides in length, respectively. The HcnB and HcnC proteins encoded 47 and 145 amino acids, respectively (Fig.20). The strain SBJ1357 was found identical in length of their deduced amino acid sequences with strains of *Pseudomonas aeruginosa*. Comparative nucleotide (*hcn*B and *hcn*C) and amino acid (HcnB and HcnC) sequence analysis showed that the SBJ1357 strain shared homologies with strains of *Pseudomonas* spp. in database. These sequences of strain SBJ1357 showed divergence at certain positions (Figs.21 and 22). However, conserved region search in the both sequences revealed the conservation of *hcn* operon in *Pseudomonas aeruginosa* strains. The *hcn*B&C genes nucleotide sequences of the SBJ1357 strain and reference strains from the NCBI database were used to determine the phylogenetic relationship. The

phylogenetic analysis revealed the distribution of the strains into five subgroups, I, II, III, IV and V (Figs.23 and 24). Strain SBJ1357 showed very close evolutionary ties with subgroup V *Pseudomonas aeruginosa* PAO1 and UCBPP-PA14 strains. The unrooted tree exhibited a small polytomy in subgroup II. To confirm the formation of these additional subgroups, a more detailed phylogenetic analysis with available sequence data was carried out to construct a complex phylogenetic tree. The topology of this tree was essentially identical with that in Figs.23 and 24. To find out any possible geographical relationship between the HCN^+ strains, the sequences of *Pseudomonas* spp. strains, whose geographical history were known either from the database or from the literature. The results established the *hcn*B&C genes geographical relationship with the strain available in database. The deduced amino acid sequences of the HcnB&C proteins of SBJ1357 strain including reference strains from the NCBI database were also used to determine the phylogenetic relationship. The detailed phylogenetic analysis based on amino acid sequences confirmed the similarity between SBJ1357 and reference HCN^+ strains (Figs.25 and 26).

Secondary structure prediction of SBJ1357 strain showed that out of HcnB 46 amino acids, 8.51 % have the propensity towards β sheet and rest (91.49 %) falls under loop region (Fig.27). In contrast to HcnB, the HcnC out of 145 amino acids have 40.69 % propensity towards alpha helix, 21.38 % forms ß-sheet and 37.93 % falls under loop region (Fig.28). Furthermore, *Pseudomonas aeruginosa* PAO1 and UCBPP-PA14 complete genome sequenced strains showed similarity in secondary structure of partial HcnB and HcnC proteins. Beside the secondary structure similarity, HcnC proteins of the strain SBJ1357 as well as other references *Pseudomonas aeruginosa* strains also showed the presence of conserved transmembrane domains at regions (7-24) and (47-64) (Fig.29).

3D-structure based fold recognition study of the HcnB protein exhibited 23 to 10 % identity and 100 to 75 % estimated precision with different Oxidoreductase (Table 3). Fold recognition search for domain identification of HcnC protein also revealed the 3D homology to Oxidoreductase with 21 and 85 % identity and estimated precision, respectively. This terminus also showed the 11 to 19 % identity and 10 to 5 % estimated precision with different Ferredoxin like enzymatic proteins (Table 4). In this study only partial *hcn*B&C genes have been sequenced, therefore, 3D models of the HcnB&C proteins of SBJ1357 strain was not carried out.

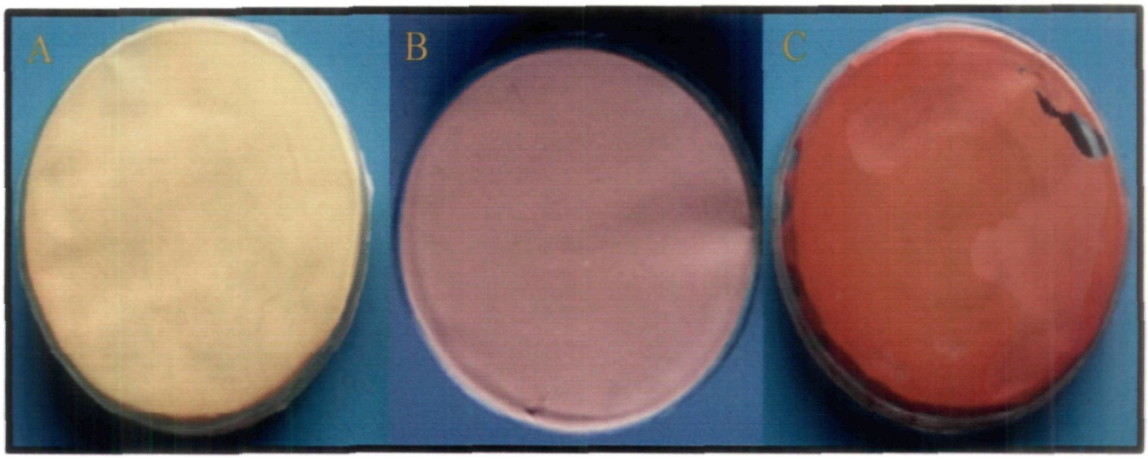


Fig.18. HCN production by strain SBJ1357.
(A) Control
(B) Absence of iron source
(C) Presence of iron source ($75\mu gml^{-1}$)

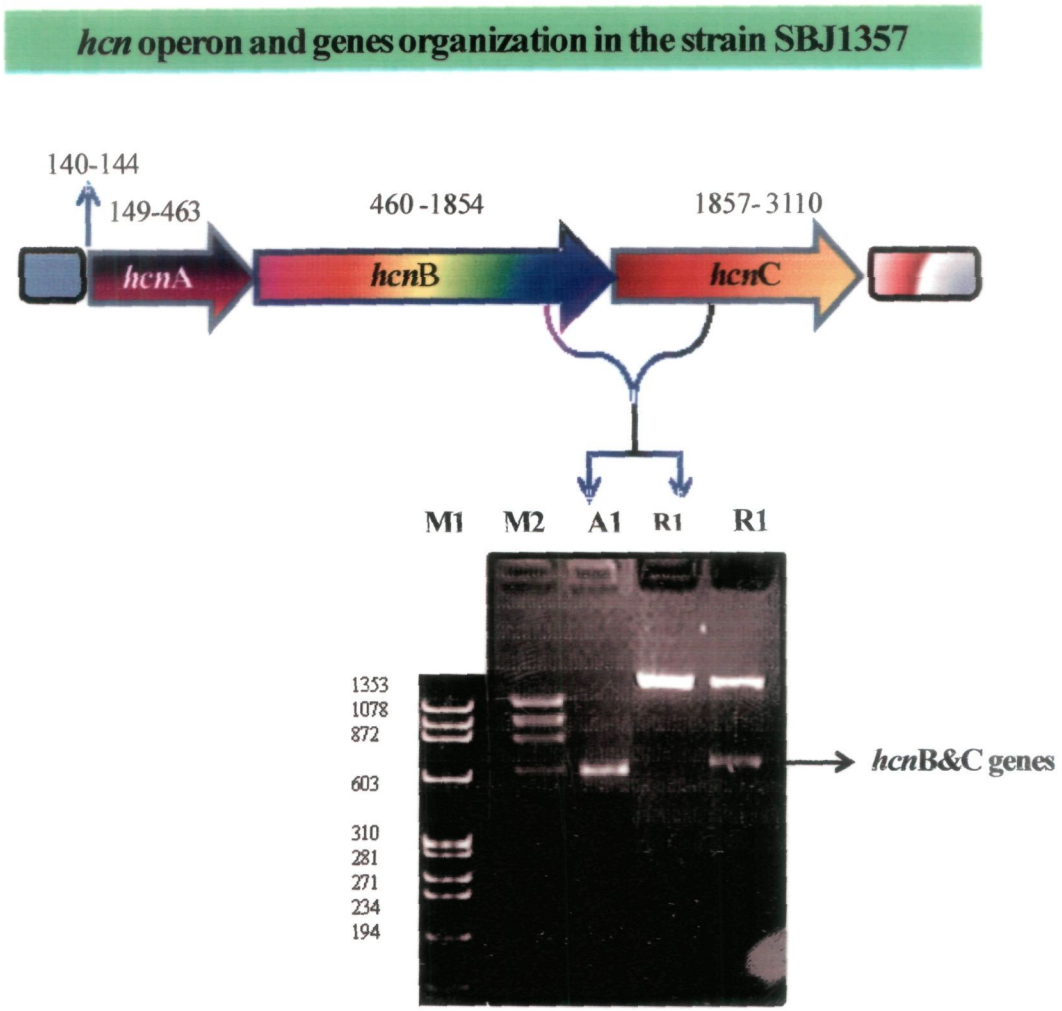


Fig.19. (A); *hcn* operon organization of the SBJ1357 and targeted genes in this study; **(B)** Agarose gel electrophoresis of the amplified *hcn*B&C genes from SBJ1357 genomic DNA

M1 and M2- □X174 *Hae* IIIDNA molecular weight marker.

A1- PCR amplified *hcn*B&C genes (0.58 kb) of the SBJ1357 strain.

R1- *Eco*RI digested plasmid vector without *hcn*B&C gene insert.

R2- *Eco*RI digested recombinant plasmid vector containing *hcn*B&C gene insert.

>*hcn*B&C genes

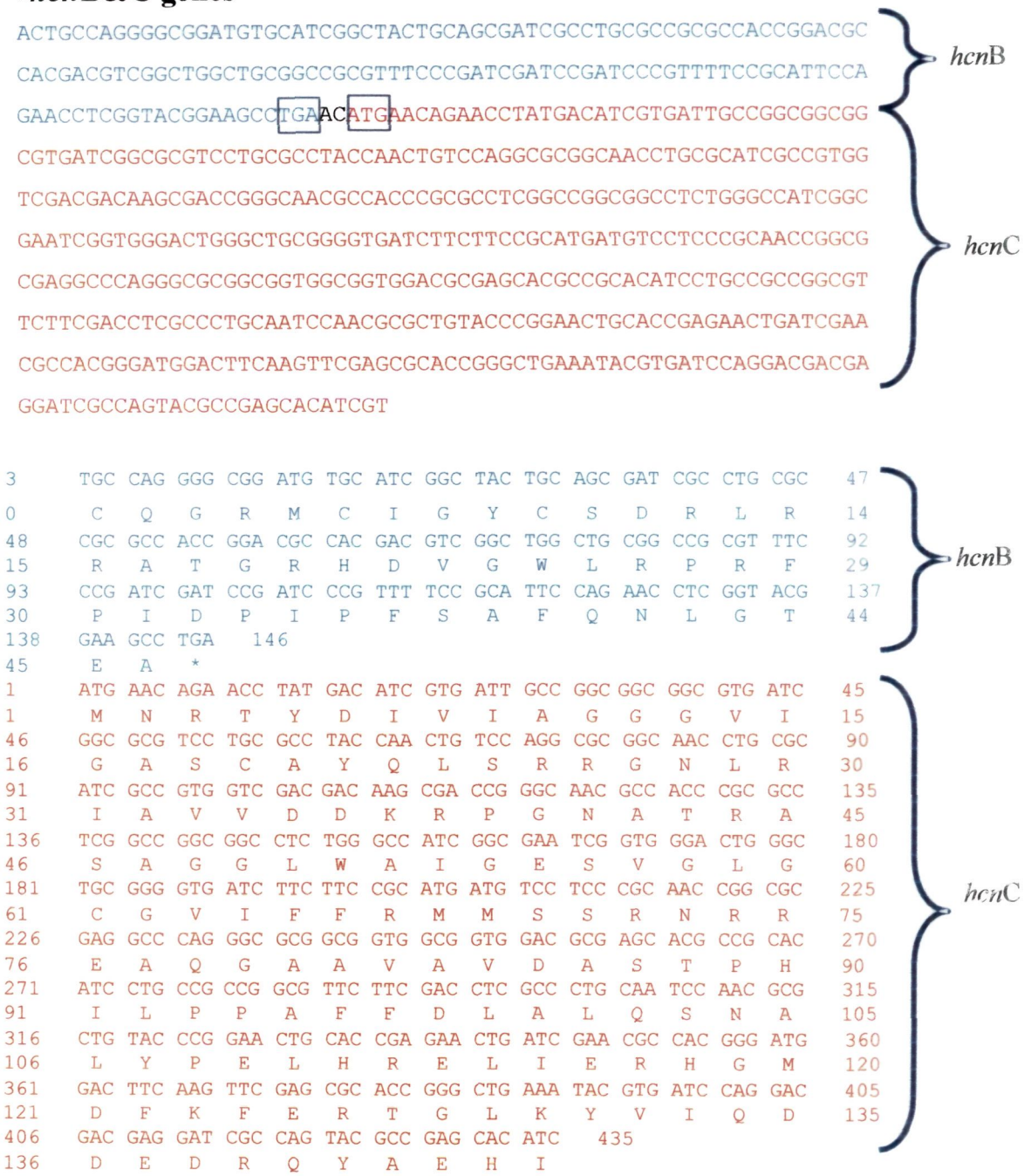


Fig.20. Nucleotide and translated amino acid sequences of the *hcn*B and *hcn*C of the strain SBJ1357. The stop codons of hcnB and start codon hcnC are depicted in box.

Table 2. Showing the nucleotide and amino acid homologies of the strain SBJ1357 related strains in NCBI database.

Type of sequence	Max. % identity	Accession No.	Min.% identity	Accession No.
***hcn*B**	100 %	*Pseudomonas aeruginosa* strain JS-11 (EF378640)	76.30 %	*Pseudomonas* sp. Strain Q7-87 (AJ418462)
HcnB	100 %	*Pseudomonas aeruginosa* (ABN80400)|	54 %	*Marinomonas* sp. MED121 (EAQ67392)|
***hcn*C**	100 %	*Pseudomonas aeruginosa* PAO1 (AE004091)	79 %	*Chromobacterium violaceum* ATCC 12472 (AE016825)
HcnC	100 %	*Pseudomonas aeruginosa* (ABN80402)|	28 %	*Marinomonas* sp. MED121 (EAQ67391)

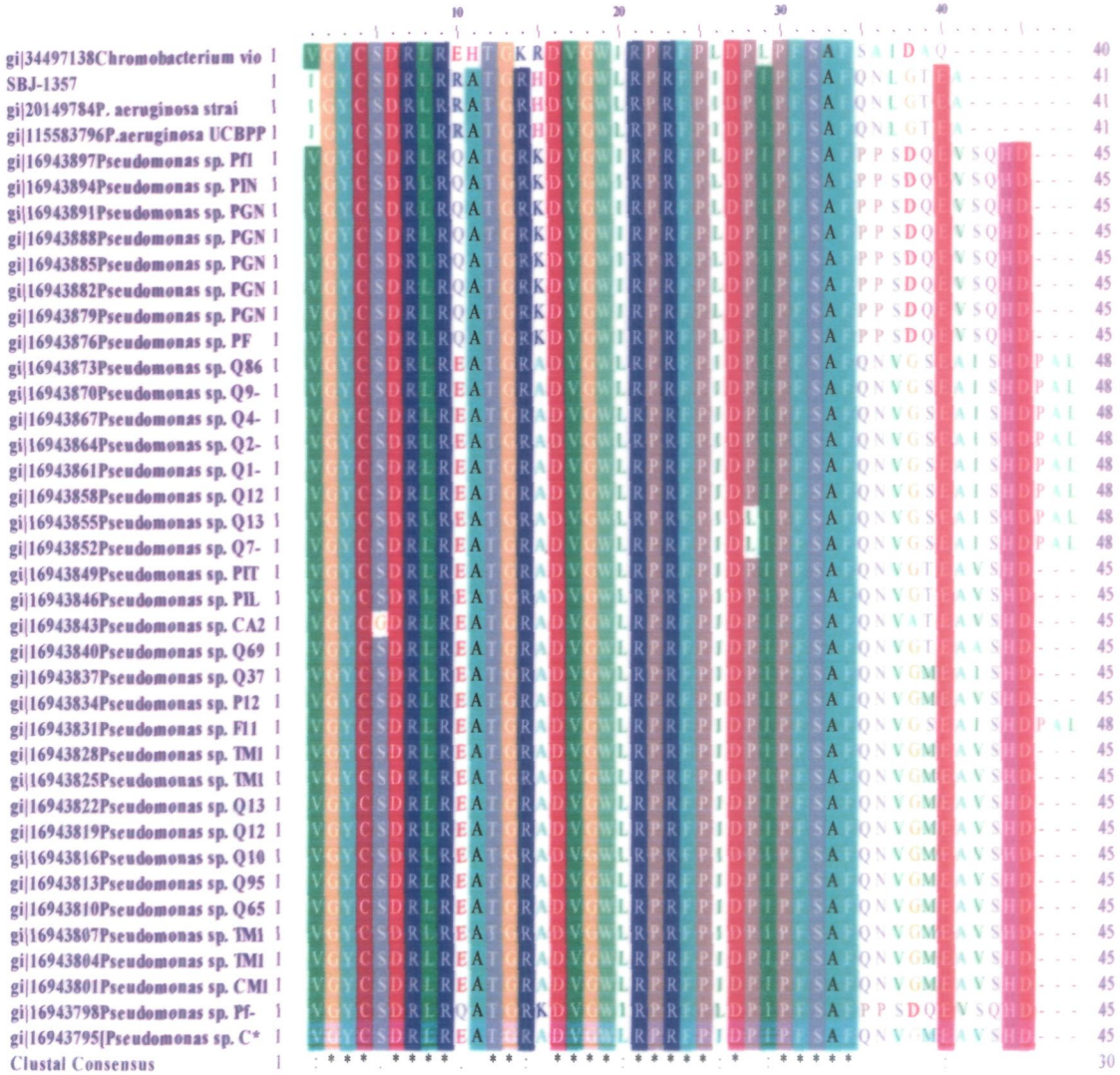
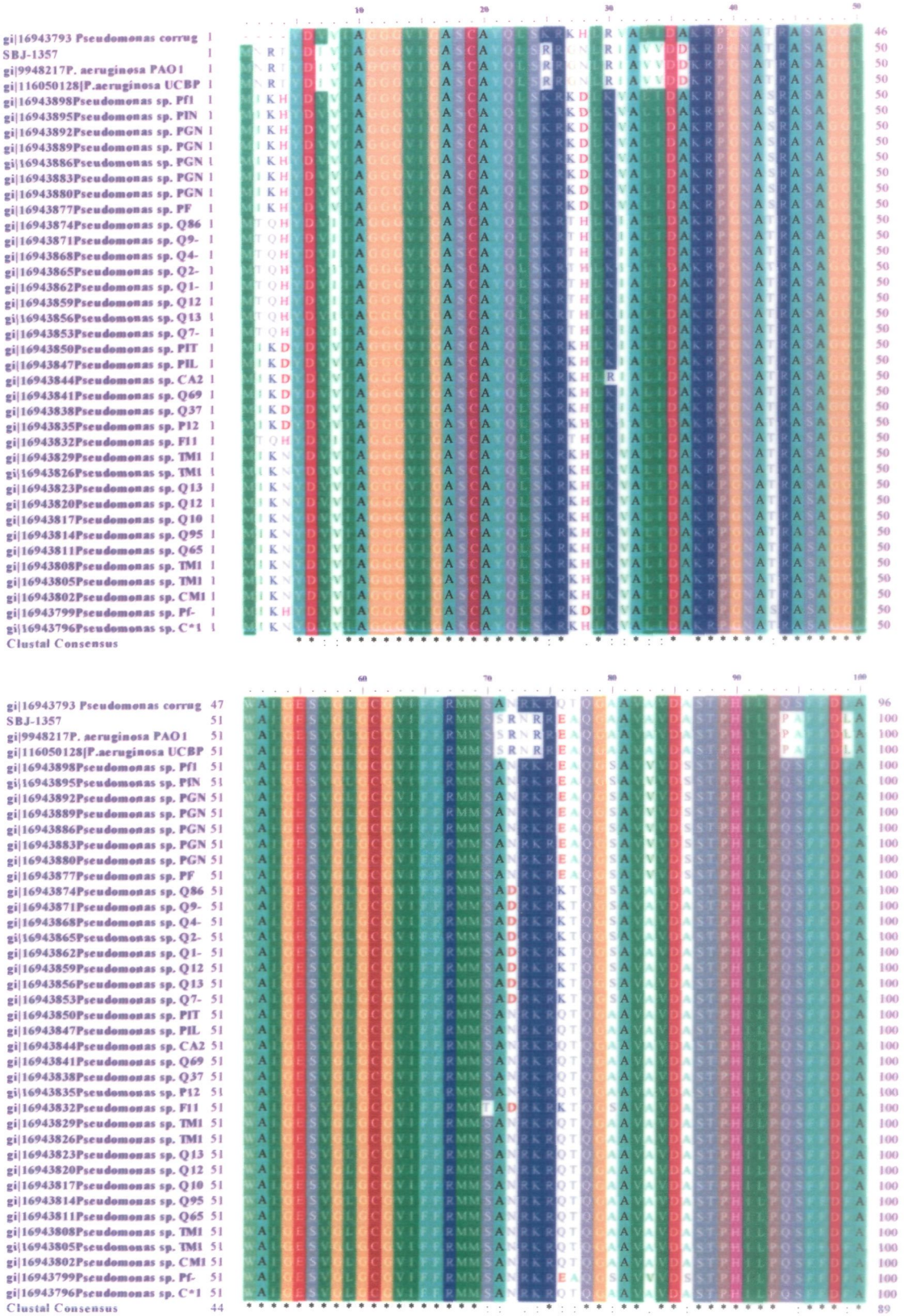


Fig.21. ClustalW alignment of HcnB protein amino acid sequence of strain SBJ1357 with reference *Pseudomonas* spp. strains.


gi|16943793 Pseudomonas corrug
SBJ-1357
gi|9948217P. aeruginosa PAO1
gi|116050128|P.aeruginosa UCBP
gi|16943898Pseudomonas sp. Pf1
gi|16943895Pseudomonas sp. PIN
gi|16943892Pseudomonas sp. PGN
gi|16943889Pseudomonas sp. PGN
gi|16943886Pseudomonas sp. PGN
gi|16943883Pseudomonas sp. PGN
gi|16943880Pseudomonas sp. PGN
gi|16943877Pseudomonas sp. PF
gi|16943874Pseudomonas sp. Q86
gi|16943871Pseudomonas sp. Q9-
gi|16943868Pseudomonas sp. Q4-
gi|16943865Pseudomonas sp. Q2-
gi|16943862Pseudomonas sp. Q1-
gi|16943859Pseudomonas sp. Q12
gi|16943856Pseudomonas sp. Q13
gi|16943853Pseudomonas sp. Q7-
gi|16943850Pseudomonas sp. PIT
gi|16943847Pseudomonas sp. PIL
gi|16943844Pseudomonas sp. CA2
gi|16943841Pseudomonas sp. Q69
gi|16943838Pseudomonas sp. Q37
gi|16943835Pseudomonas sp. P12
gi|16943832Pseudomonas sp. Fl1
gi|16943829Pseudomonas sp. TM1
gi|16943826Pseudomonas sp. TM1
gi|16943823Pseudomonas sp. Q13
gi|16943820Pseudomonas sp. Q12
gi|16943817Pseudomonas sp. Q10
gi|16943814Pseudomonas sp. Q95
gi|16943811Pseudomonas sp. Q65
gi|16943808Pseudomonas sp. TM1
gi|16943805Pseudomonas sp. TM1
gi|16943802Pseudomonas sp. CM1
gi|16943799Pseudomonas sp. Pf-
gi|16943796Pseudomonas sp. C*1
Clustal Consensus

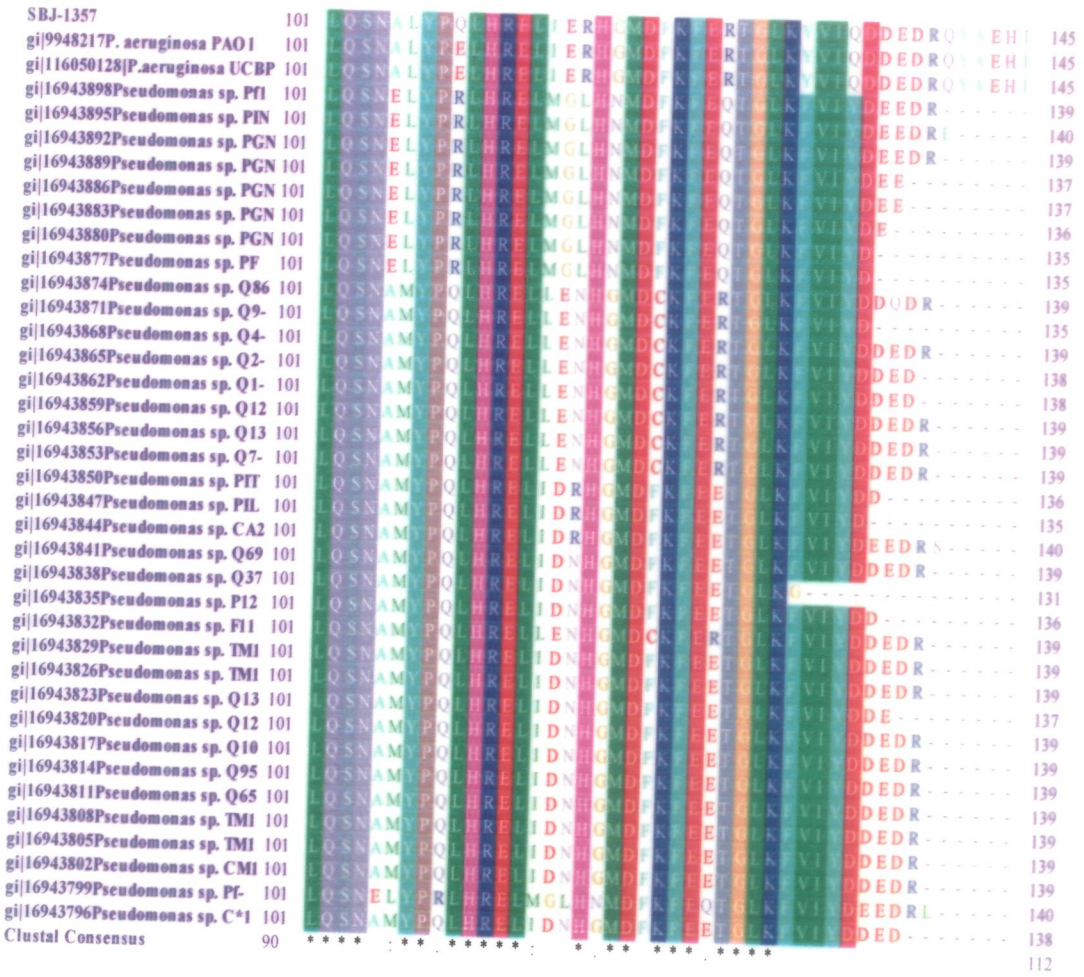


Fig.22. ClustalW alignment of HcnC protein amino acid sequence of strain SBJ1357 with reference *Pseudomonas* spp. strains.

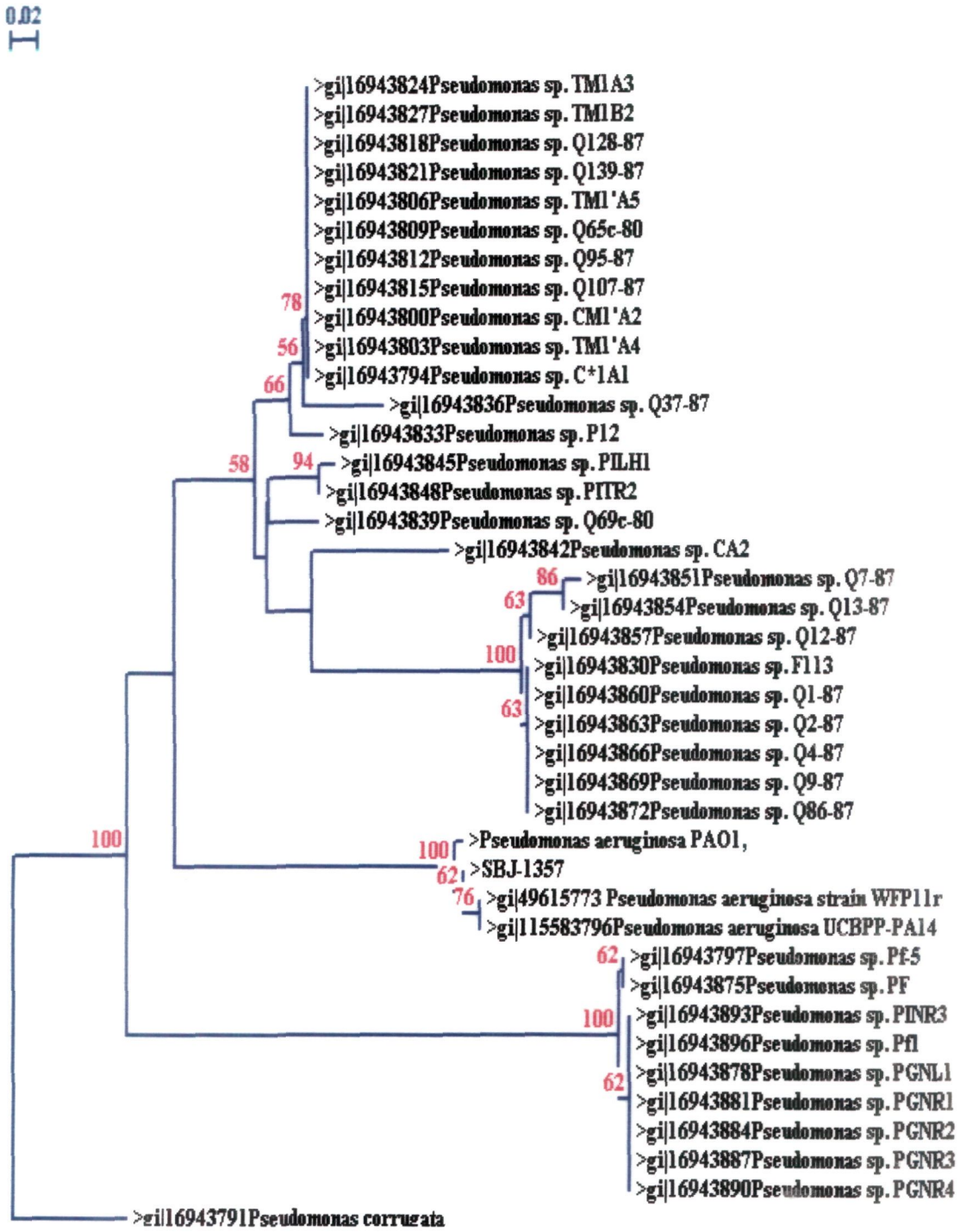


Fig.23. Phylogenetic relationship based on *hcn*B nucleotide sequences of SBJ1357 and reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40 % or greater are indicated on the tree. The scale bar indicates the numbers of nucleotides substitutions per site.

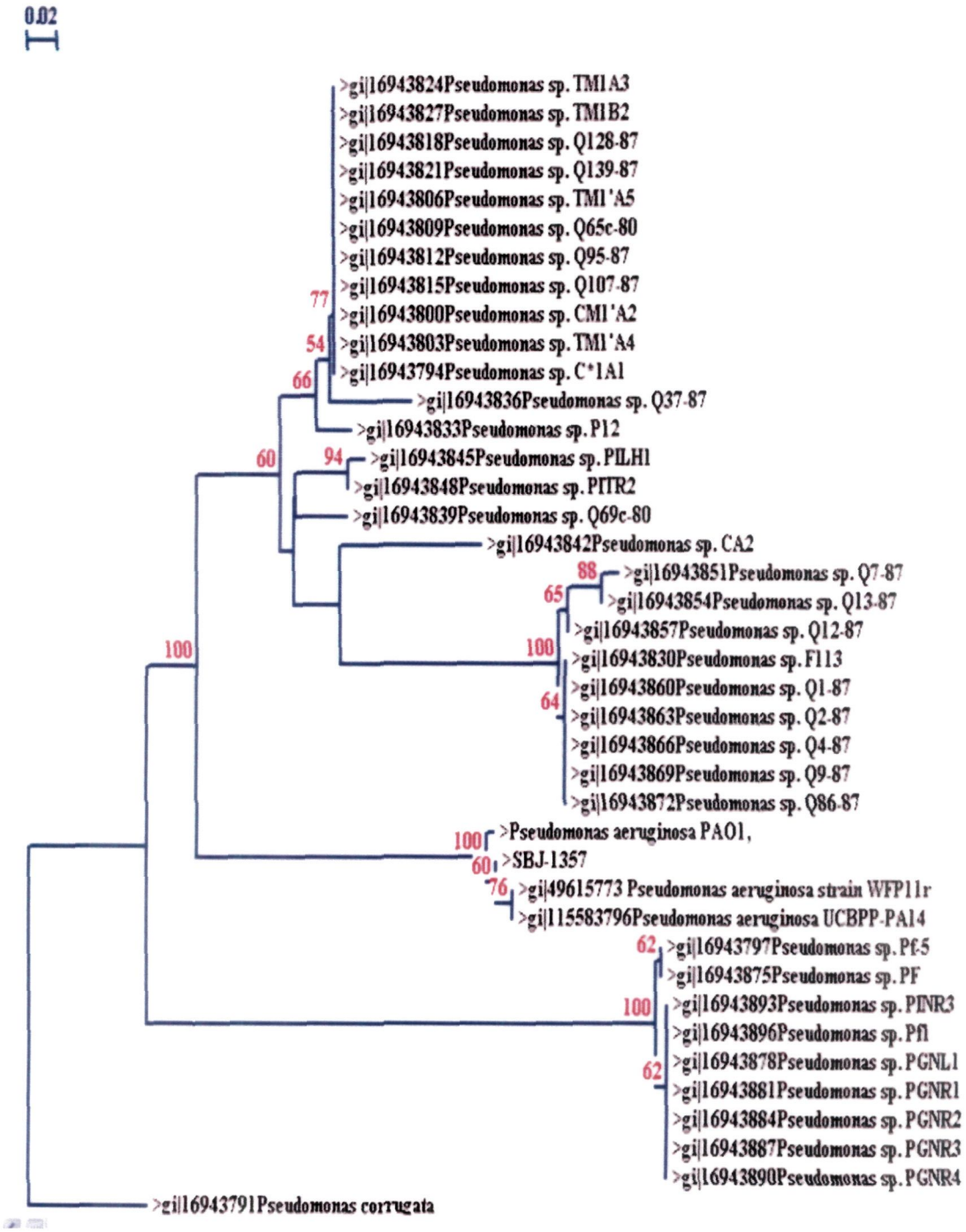


Fig.24. Phylogenetic relationship based on *hcn*C nucleotide sequences of SBJ1357 and reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40 % or greater are indicated on the tree. The scale bar indicates the numbers of nucleotides substitutions per site.

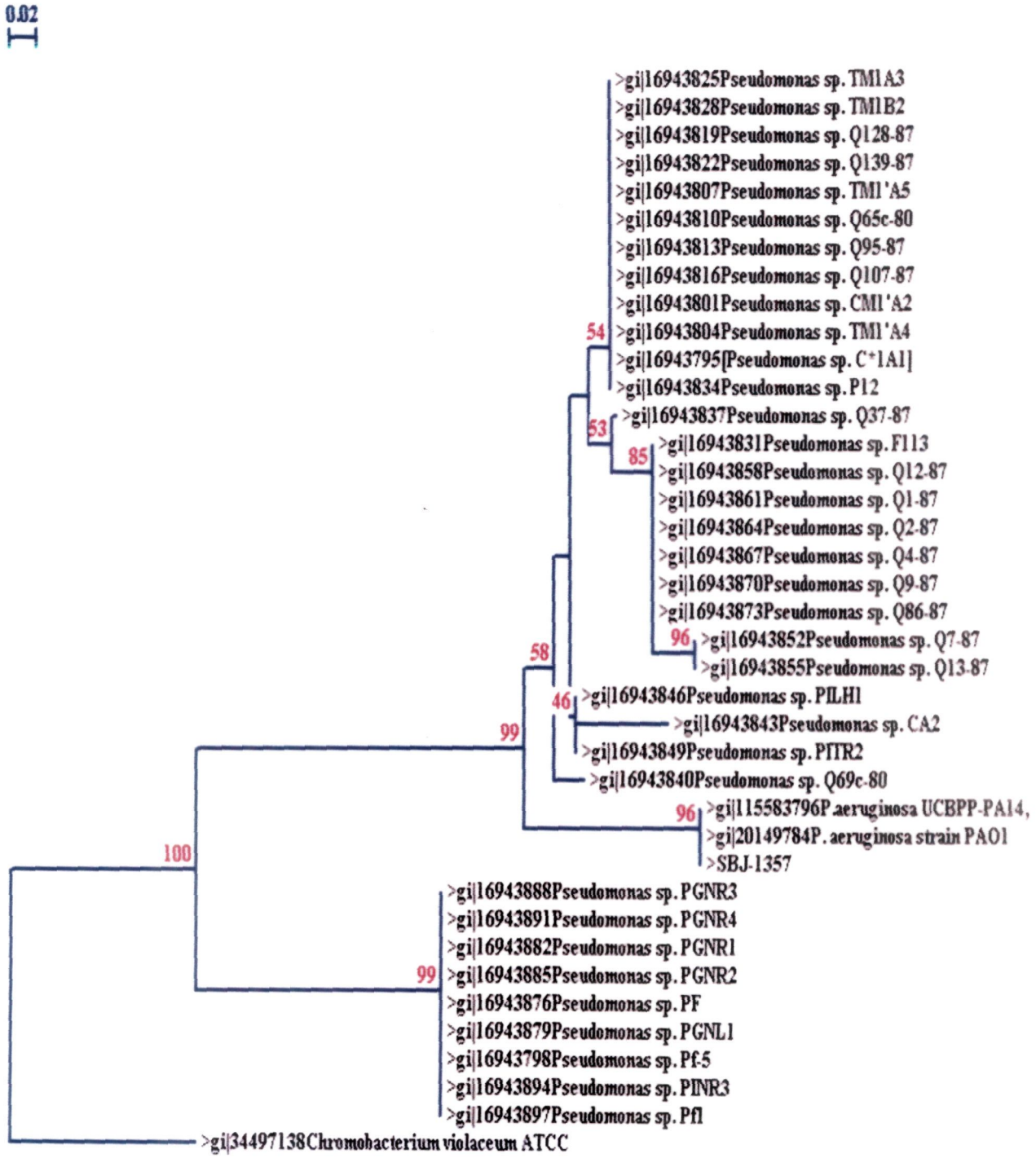


Fig.25. Phylogenetic relationship based on HcnB amino acid sequences of SBJ1357 and reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40 % or greater are indicated on the tree. The scale bar indicates the numbers of amino acid substitutions per site.

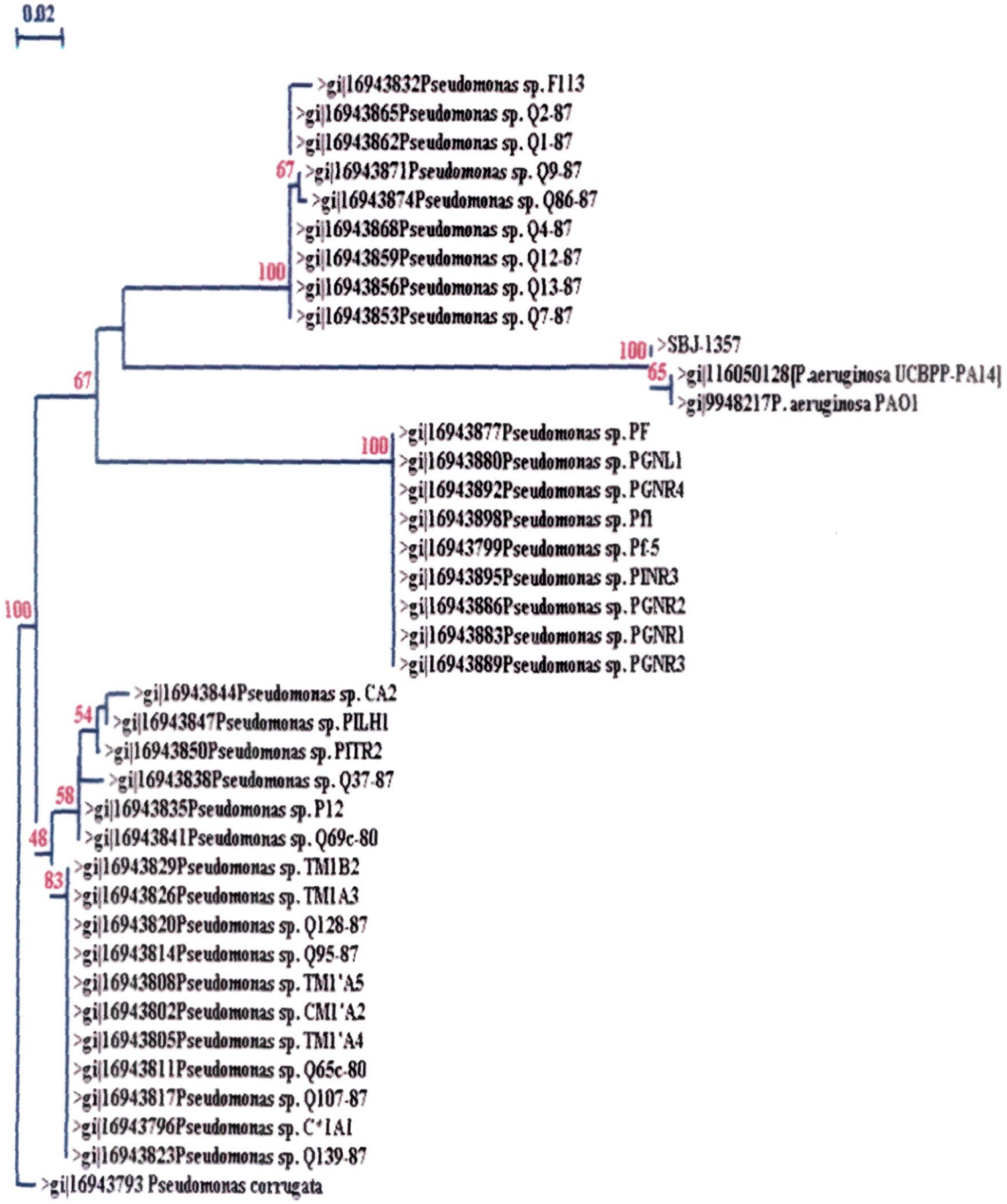


Fig.26. Phylogenetic relationship based on HcnC amino acid sequences of SBJ1357 and reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40 % or greater are indicated on the tree. The scale bar indicates the numbers of amino acid substitutions per site.

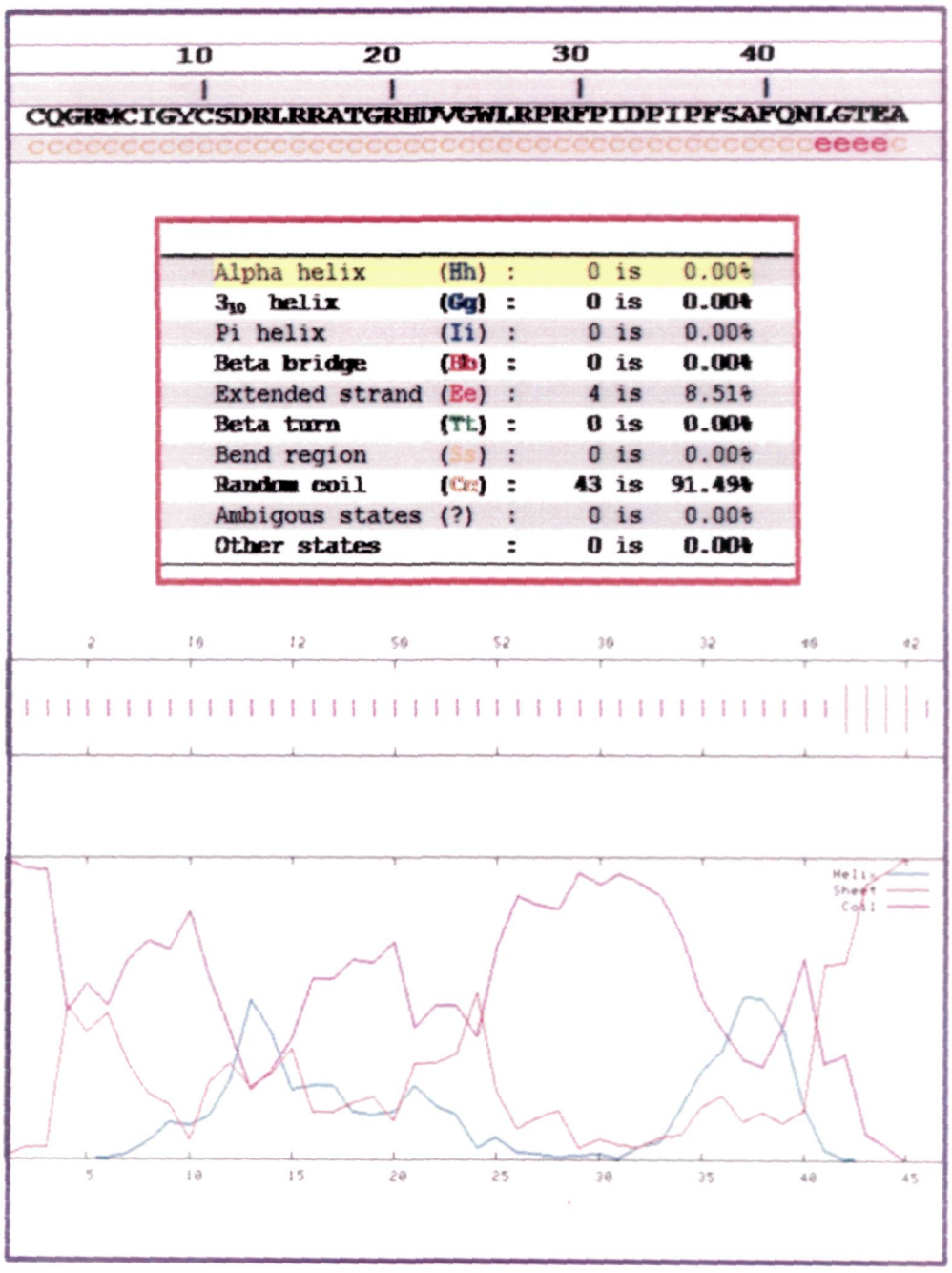


Fig.27. Secondary structure of the HcnB protein of strain SBJ1357.

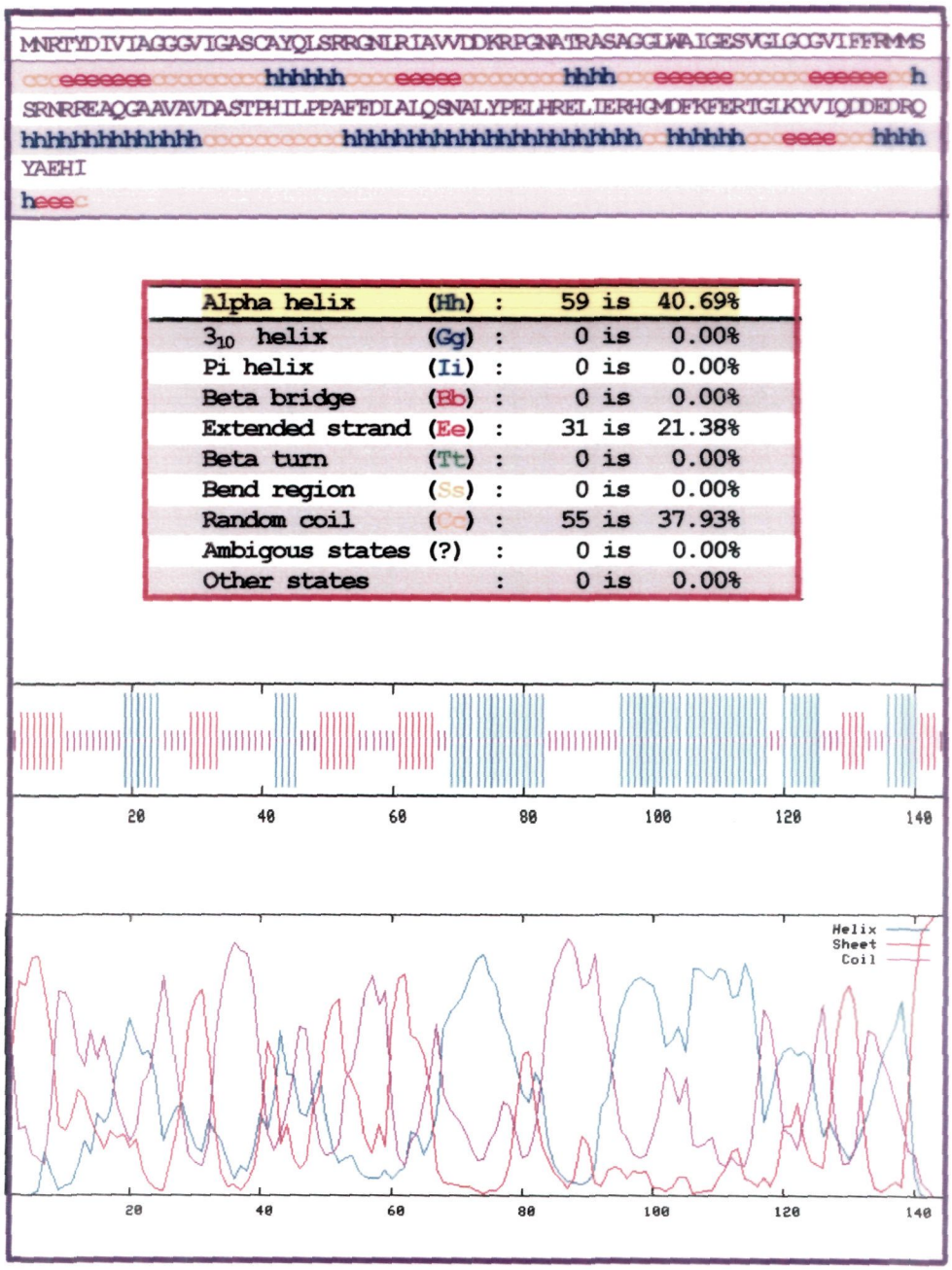


Fig.28 . Secondary structure of the HcnC protein of strain SBJ1357.

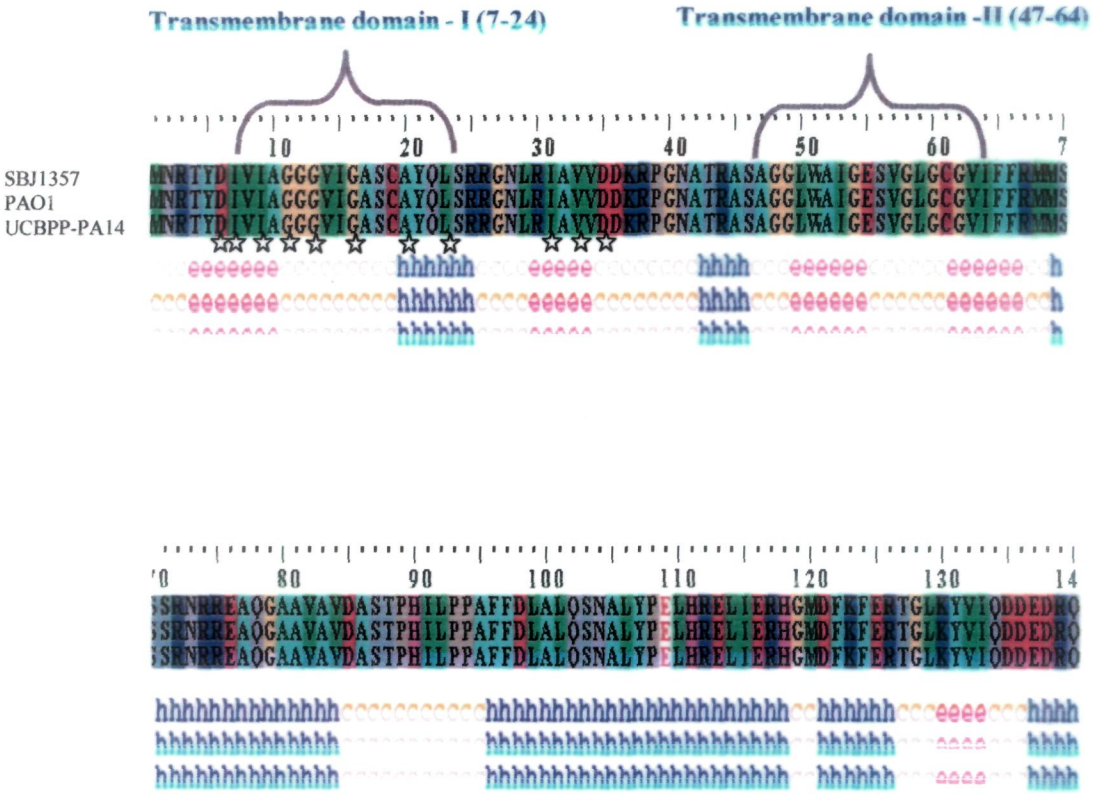


Fig.29. Alignment of amino acid sequences and secondary structure of the HcnC protein of strain SBJ1357. The N-terminal of the HcnC protein of SBJ1357 shows the presence of transmembrane domains. The 11 key residues (☆) of HcnC protein correspond to the sequence fingerprint of the ADP-binding fold (Wierenga *et al.*, 1986).

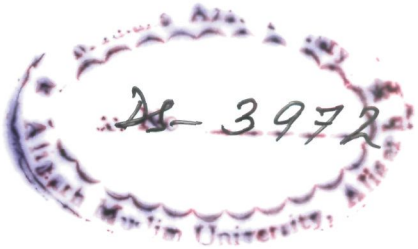
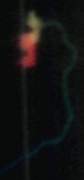
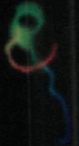


Table.3. 3D-fold recognition ranking and alignments of the HcnB protein of strain SBJ1357.

SCOP Code	Estimated Precision	Fold/PDB descriptor	Superfamily	Family	Models
c1y56A (length:493) 21% i.d.	85%	oxidoreductase	hypothetical protein ph1363;	crystal structure of l-proline dehydrogenase from2 p.horikoshii	
d2nef (length:136) 17% i.d.	10%	Regulatory factor Nef	Regulatory factor Nef	Regulatory factor Nef	
d1uj6a2 (length:74) 19% i.d.	5%	Ferredoxin-like	D-ribose-5-phosphate isomerase (RpiA), lid domain	D-ribose-5-phosphate isomerase (RpiA), lid domain	
d1hyoa1 (length:118) 11% i.d.	5%	SH3-like barrel	Fumarylacetoacetate hydrolase, FAH, N-terminal domain	Fumarylacetoacetate hydrolase, FAH, N-terminal domain	
c2oc6A (length:124) 11% i.d.	5%	structural genomics, unknown function	ydhg protein;	crystal structure of a protein of unknown function from2 cog5646 family (np_388456.1) from bacillus subtilis at3 1.75 a resolution	
d1lk5a2 (length:80) 11% i.d.	5%	Ferredoxin-like	D-ribose-5-phosphate isomerase (RpiA), lid domain	D-ribose-5-phosphate isomerase (RpiA), lid domain	

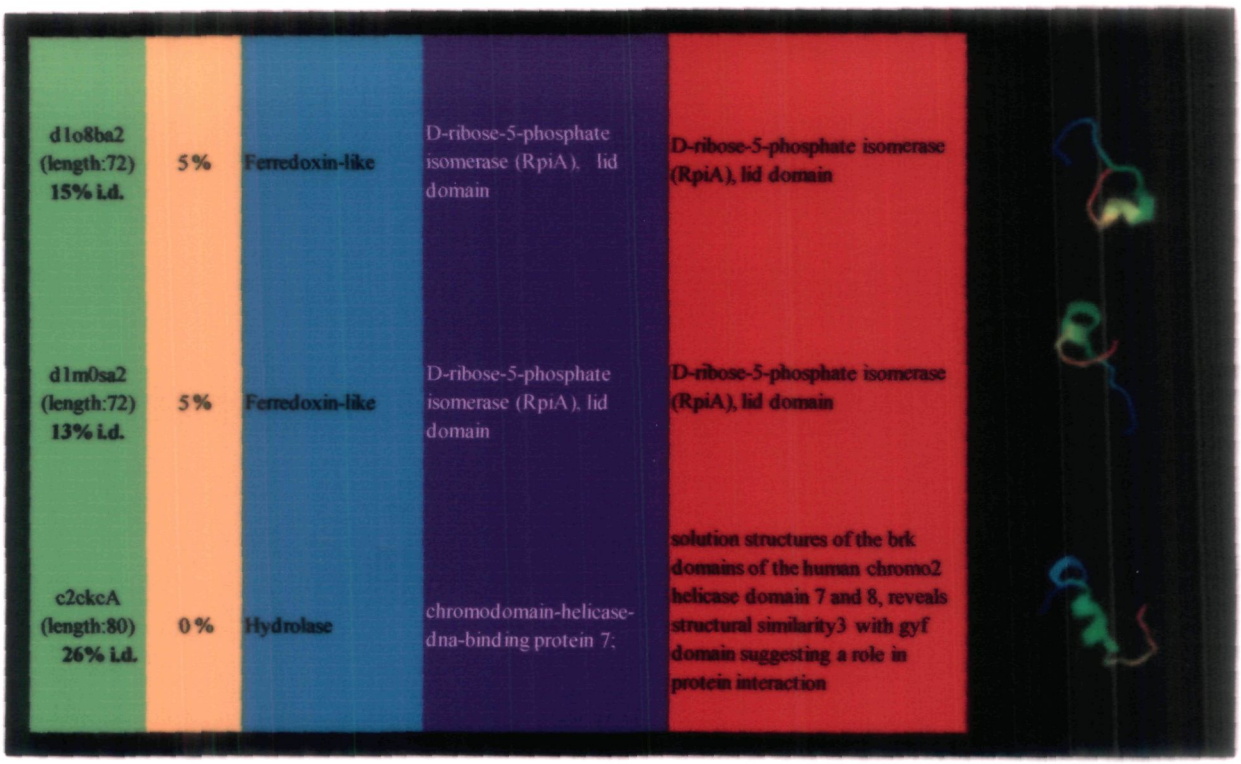
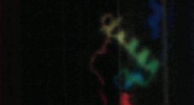
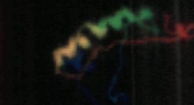
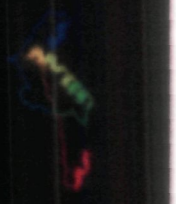

d1o8ba2 (length:72) 15% i.d.
5%
Ferredoxin-like
D-ribose-5-phosphate isomerase (RpiA), lid domain
D-ribose-5-phosphate isomerase (RpiA), lid domain
d1m0sa2 (length:72) 13% i.d.
5%
Ferredoxin-like
D-ribose-5-phosphate isomerase (RpiA), lid domain
D-ribose-5-phosphate isomerase (RpiA), lid domain
c2ckcA (length:80) 26% i.d.
0%
Hydrolase
chromodomain-helicase-dna-binding protein 7;
solution structures of the brk domains of the human chromo2 helicase domain 7 and 8, reveals structural similarity3 with gyf domain suggesting a role in protein interaction

Table.4. 3D-fold recognition ranking and alignments of the HcnC protein of strain SBJ1357.

SCOP Code	Estimated Precision	Fold/PDB descriptor	Superfamily	Family	Models
d1ng4a1 (length:277) 23% i.d.	100 %	FAD/NAD(P)-binding domain	FAD/NAD(P)-binding domain	FAD-linked reductases, N-terminal domain	
c1ng3A (length:390) 23% i.d.	100 %	oxidoreductase	glycine oxidase:	complex of thio (glycine oxidase) with acetyl-glycine	
c2gagB (length:405) 12% i.d.	95 %	oxidoreductase	heterotetrameric sarcosine oxidase beta-subunit:	heterotetrameric sarcosine: structure of a diflavin2 metaloenzyme at 1.85 a resolution	
c1y56B (length:382) 12% i.d.	95 %	oxidoreductase	sarcosine oxidase:	crystal structure of l-proline dehydrogenase from2 p.horikoshii	
d1pj5a2 (length:306) 11% i.d.	95 %	FAD/NAD(P)-binding domain	FAD/NAD(P)-binding domain	FAD-linked reductases, N-terminal domain	
d1el5a1 (length:282) 12% i.d.	95 %	FAD/NAD(P)-binding domain	FAD/NAD(P)-binding domain	FAD-linked reductases, N-terminal domain	

c1pj5A (length:830) **12% i.d.**	95 %	oxidoreductase	n,n-dimethylglycine oxidase;	crystal structure of dimethylglycine oxidase of2 arthrobacter globiformis in complex with acetate	
c2oloA (length:397) **17% i.d.**	95 %	oxidoreductase	nikd protein;	nikd, an unusual amino acid oxidase essential for2 nikkomycin biosynthesis: open form at 1.9a resolution	
c1zovA (length:386) **10% i.d.**	95 %	oxidoreductase	monomeric sarcosine oxidase;	crystal structure of monomeric sarcosine oxidase from2 bacillus sp. ns-129	

3.3.1.3. Assessment of phenazine-1-carboxylic acid (PCA) production by SBJ 1357and sequencing and *In-Silico* analysis of *phz*C,D,E&S genes.

PCA production of strain SBJ1357 was monitored at different time points in LB medium amended with 30 mM glucose. The production of PCA started at the late exponential phase reached its maximum at the stationary phase (Fig.30). The PCA was quantitated spectrophotometrically measuring the absorbance of yellow at 367 nm, in benzene extract of the culture (Fig.31A). The extract was also analysed by HPLC using C-18 Novapak (5μm) column with mobile phase of acetonitrile : water (70:30) at 254 nm and the peak of PCA was noticed (Fig.31B). Identification of cultural conditions with controlled PCA production will lead to a better understanding of regulation of the biosynthesis of this secondary metabolite. This information may become important in view of optimizing the biocontrol activity of the strain under practical conditions. Therefore, the effects of certain abiotic factors on PCA production by strain SBJ1357 have also been investigated. In order to study the effects of various carbon sources on PCA production, the strain SBJ1357 was grown in minimal salt medium supplemented with 0.5 mM and 1.0 mM each of carbon sources *viz.* glucose, sucrose, fructose, lactose and citric acid independently. Absorbance at stationary phase was determined. Strain SBJ1357 has not shown in sign of growth on the citric acid. The carbon sources, glucose and fructose showed the highest PCA production, while lactose and sucrose supported moderate PCA production (Fig.32). The PCA was also severely influenced by the change in pH and temperature.

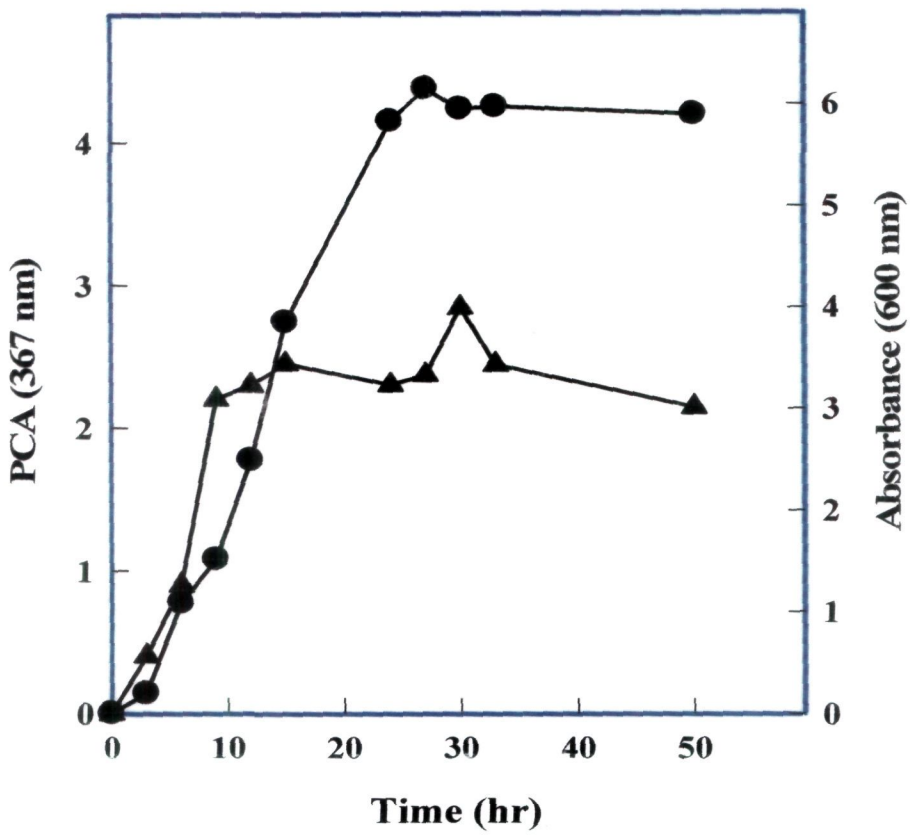
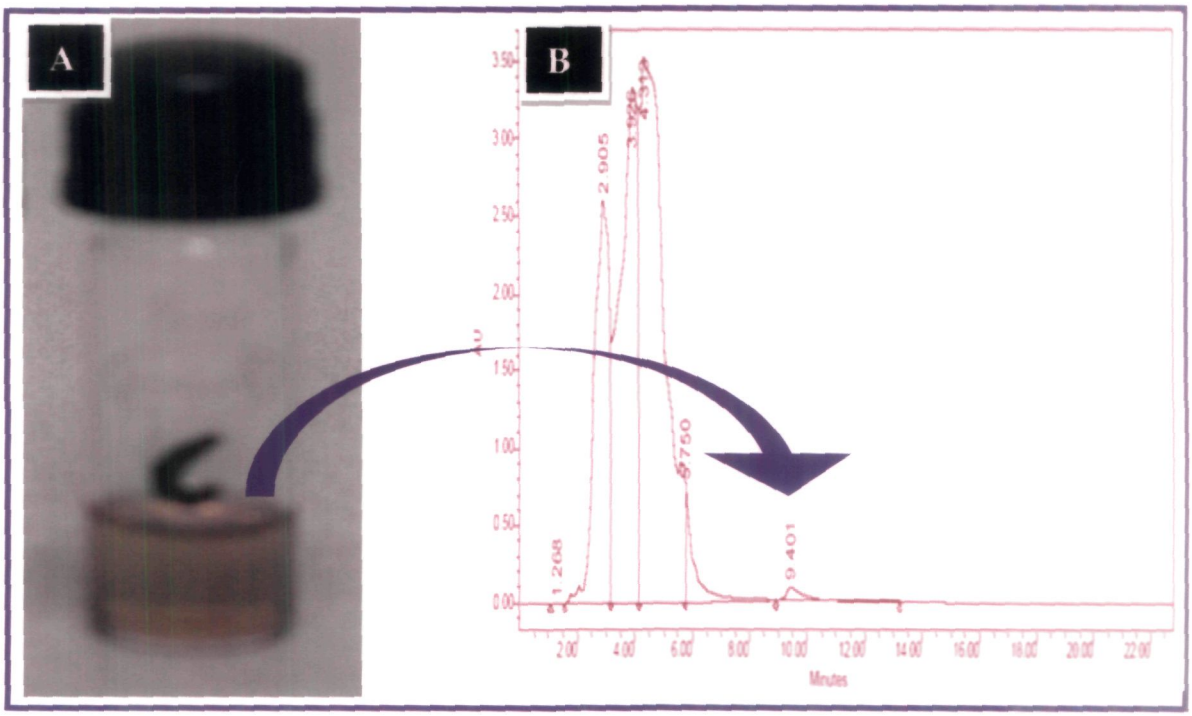


Fig.30. Growth and PCA production by strain SBJ-1357. Cells were grown on LB medium amended with 30 mM glucose. Amounts of PCA were determined by UV-visible spectrophotometer. Experiments were performed three times with similar results. Data of one of the experiments is shown.



Figs.31. (A) Benzene extrct showing the presense of PCA (yellow colour)
(B) HPLC profile of PCA at retention time 9.5 on C-18 Novapack (5μM) column, using mobile phase (acetonitrile:water, 70:30). Absorption was read at 254 nm.

The data revealed that PCA production decreases in the range from pH 6.0 to pH 7.5. However, it increases again in alkaline range of pH 8.0 and above up to pH 9.0 (Fig.33). Temperature is also an important factor under field conditions and may influence PCA production severely. Testing of PCA production at temperatures of 28 °C, 32 °C and 37 °C showed that PCA production is directly proportional to increase in temperature (Figs.34 and 35). Also, the color of culture supernatant changed from yellow to green as the temperature possibly due to increased accumulation of pyocyanin, (phenazine derivative) (Fig.36). From a practical perspective, the effect of mineral on PCA biosynthesis may explain the association between soil chemical and physical properties and the variable performance of antagonistic activities of strains between field sites. Assessment of the relationship between $ZnSO_4$ and PCA production at varying concentration of $ZnSO_4$ (0 - 55 μgml^{-1}) suggested a positive relationship. Maximum PCA production was noticed at $ZnSO_4$ concentration 35 μgml^{-1}. Any further increase in $ZnSO_4$ level resulted in the reduction in PCA production (Fig.37). Interestingly, the substitution of exogenous amino acids in the medium for PCA production appears to be indispensible. The strain SBJ1357 produced hardly any PCA when grown in minimal salt medium without amino acids. The strain was also screened for AHL production using cross-feeding test. A positive reaction was recorded for strain SBJ1357 (Fig.38). It can be seen that the incubation of SBJ1357 in the presence of the reporter strain, *A. tumefaciens* A136 [(Ti) (pCF218) (pCF372)], produced a blue coloration (hydrolysis of chromogeneic substrate X-gal) in the bioassay medium due to the expression of the *lacZ* reporter gene. The genetic element pCF 218 codes for TraR protein (AHL responsive transcription factor that recognized wide range of related AHLs) and TraR regulated *tra*I-*lac*Z fused reporter are carried on pCF372.

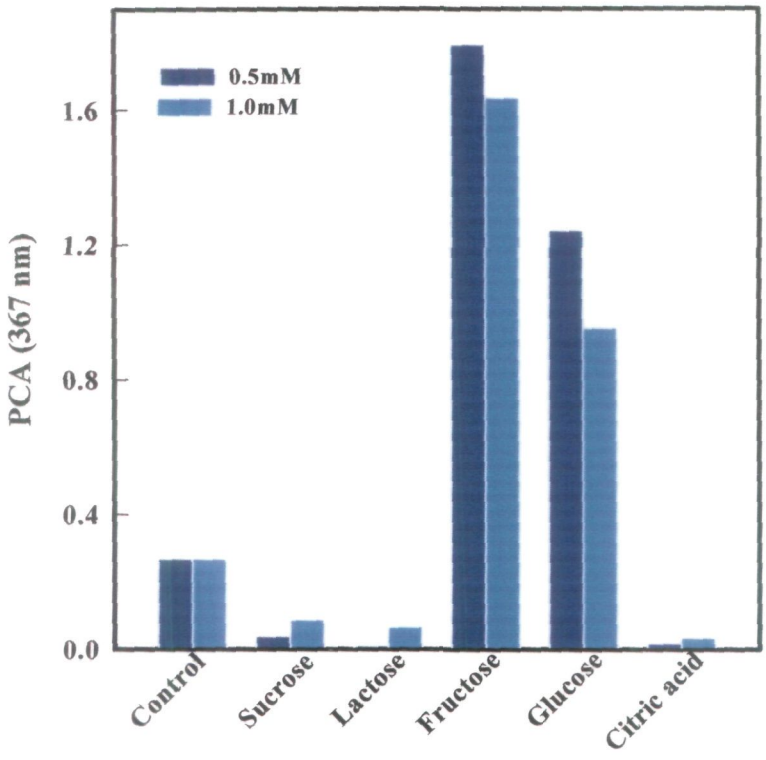


Fig.32. Influence of various carbon sources on the level of PCA production by strain SBJ-1357. The strain was grown in minimal salt medium amended with various carbon sources at 0.5 mM and 1.0 mM concentrations.

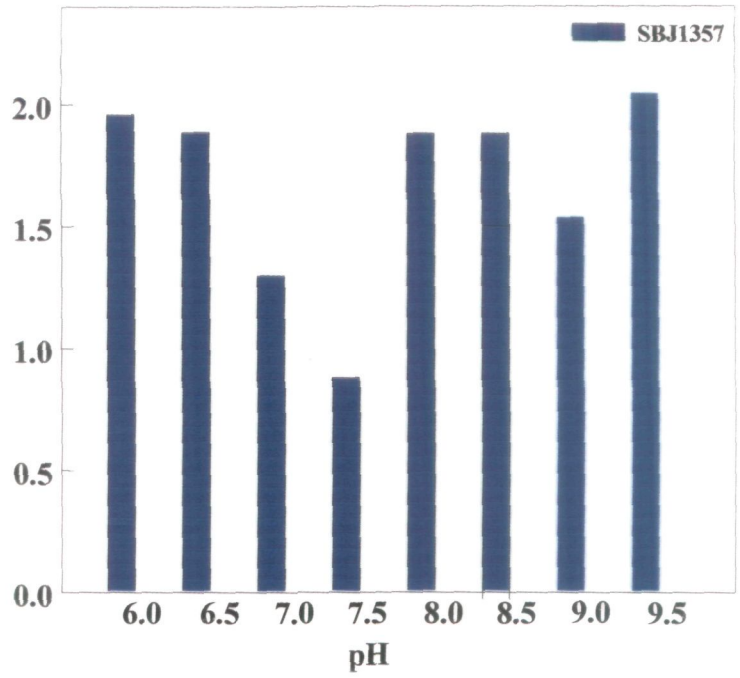


Fig.33. Effect of pH on PCA production by strain SBJ1357

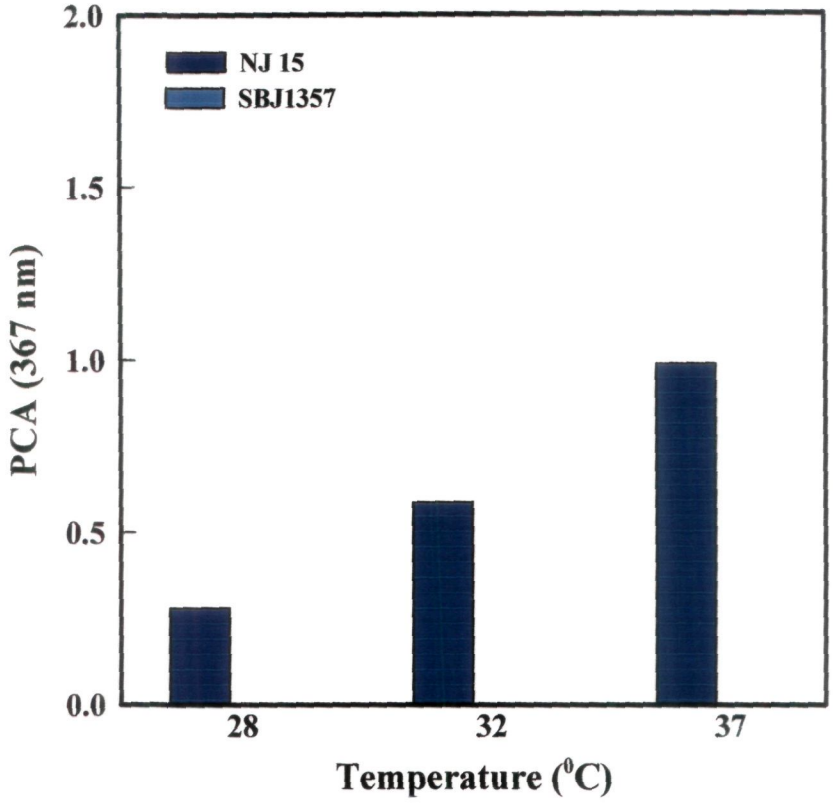
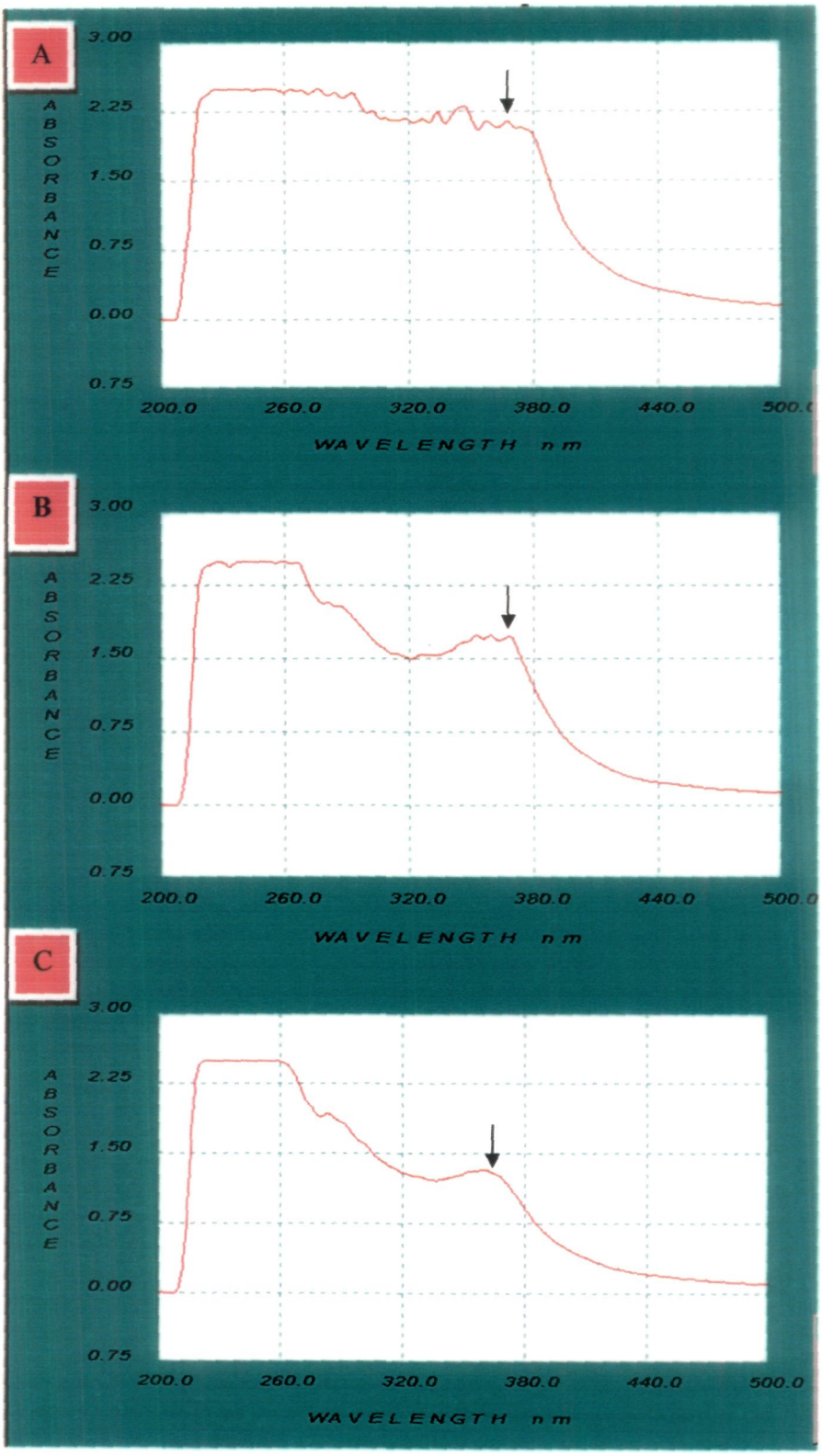


Fig.34. Effect of temperature on PCA production by strain SBJ1357. The strain was grown in minimal salt medium amended with 0.05 % casamino acids and 1.5% (w/v) glucose.



Figs.35. (A), (B) and (C); UV-visible spectra showing the temperature dependent production of the PCA by strain SBJ1357 at 37°C, 32 °C and 28°C, respectively.

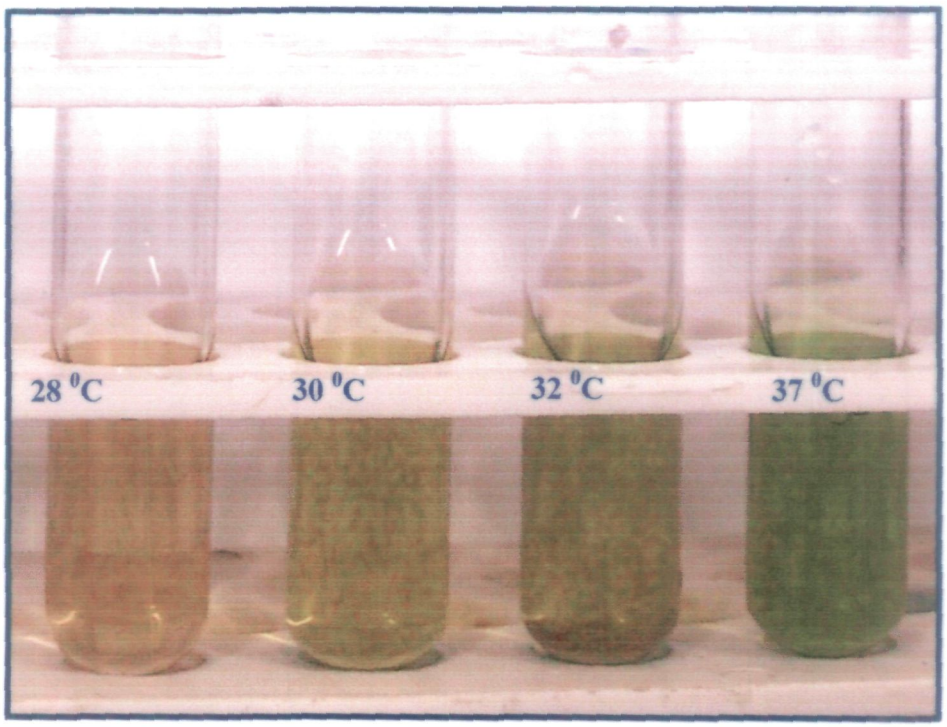


Fig.36. Effect of temperature on the production of phenazine metabolites by strain SBJ1357.

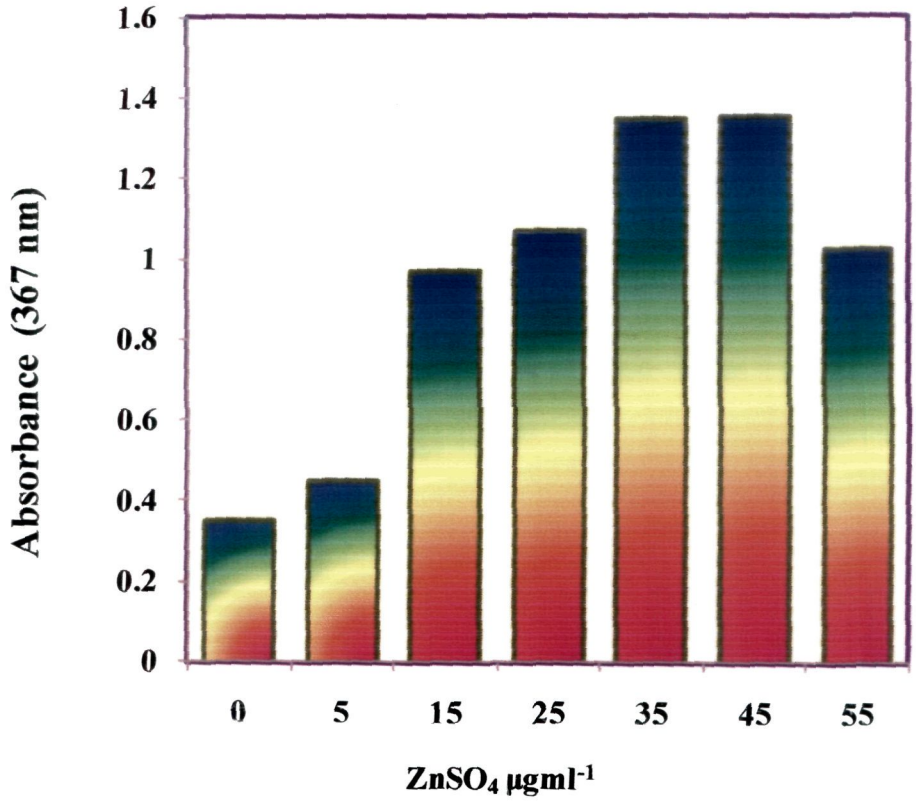


Fig.37. Influence of various concentrations of $ZnSO_4$ (0, 5, 15, 25, 35, 45 and 55 μgml^{-1}) on PCA production by strain SBJ1357.

Fig.38. The cross feeding assay showing the production of AHLs as evident by the expression of β-galactosidase activity (blue coloration) in the reporter strain, *A. tumefaciens* A136, which is streaked across the top half of the plate. The (A) and (B) Shows production of AHLs by strains SBJ1357 and NJ101, respectively.

A similar reaction was observed when the AHL-producing strain *P. aeruginosa* NJ101 was cultivated with *A. tumefaciens* A136 (positive control). The data confirmed the direct relationship of PCA production with quorum sensing (cell density) (Fig.30)

The PCA producing ability of strain SBJ1357 also has been confirmed by molecular analysis using molecular biology and bioinformatics tools. The strain SBJ1357 does contain the *phz* operon was validated by the targeting *phz*C,D,E&S genes. These genes from SBJ1357 were successfully amplified by self-designed gene specific primers by PCR (Fig.39A). The amplicons of 1405, 1480 and1499 bp were obtained (Fig.39B). The amplicons were successfully cloned in plasmid cloning vector and presence of the desired genes inserts in recombinant clones were validated through PCR and restriction analysis. The restriction digestion of clones with the inserts of *phz*C&D and *phz*D&E genes suggested the absence of internal *Eco*R I sites. However, the insert of phzS genes exhibited the presence of single internal *Eco*R I site (Fig.39B). The positive clones were sequenced, and blast analysis performed. The *phz*C,D,E&S genes of strain SBJ1357 showed the highest sequence homology with the *Pseudomonas aeruginosa* strains reported in public database. The sequenced *phz* C,D,E&S genes were translated into amino acid sequences which were found to be of 274, 207, 202 and 201 amino acids in length, respectively (Figs. 40 to 44). Comparative nucleotide (*phz*C,D,E&S) and amino acid (PhzC,D,E,&S) sequences analysis showed that the strain SBJ1357 shared variable identities with all *Pseudomonas aeruginosa* and other bacterial strains. The *phz*C,D,E&S genes of the strain SBJ1357 were found identical in length of their deduced amino acid sequences in comparison with *Pseudomonas aeruginosa* strains their genome complete sequenced. However, the variability and divergence was noticed between the different species of the same genus as well as different genus (Figs. 45 to 48).

The Phz C,D,E,&S amino acid sequences of the strain SBJ1357and reference strains from the NCBI database were used to determine the phylogenetic relationship. The phylogenetic analysis revealed the distribution of the strains in to different groups (Figs.49 and 50). The data revealed a very close evolutionary ties of strain SBJ1357 with the group of *Pseudomonas aeruginosa* PAO1 and UCBPP-PA14 strains. The unrooted trees showed a small polytomy in some clads. To confirm the formation of these additional subgroups, a more detailed phylogenetic analysis with available sequence data was carried out to construct a complex phylogenetic tree. The results showed that the PhzC&D proteins have

some extent geographical relationship. The phylogenetic analysis based on PhzE&S amino acid sequences further confirmed similar existence of SBJ1357 in relation to the reference *Pseudomonas aeruginosa* strains (PAO1 and UCBPP-PA14).

PhzC, D, E & S secondary structure prediction of SBJ1357 strain have also been performed. The 274 amino acids of PhzC exhibit 45.62% propensity towards α helix and rest 10% extended and remaining 44.16% falls under loop region (Fig.51.). In contrast to PhzC, the PhzD revealed that out of 207 amino acids, 51.21 % has propensity towards α helix, 13.83 % extended strand 35.27 % falls under loop region (Fig.52). The *Pseudomonas aeruginosa* PAO1 and UCBPP-PA14 complete genome sequenced strains also showed similarity with the secondary structures of PhzC&D proteins.

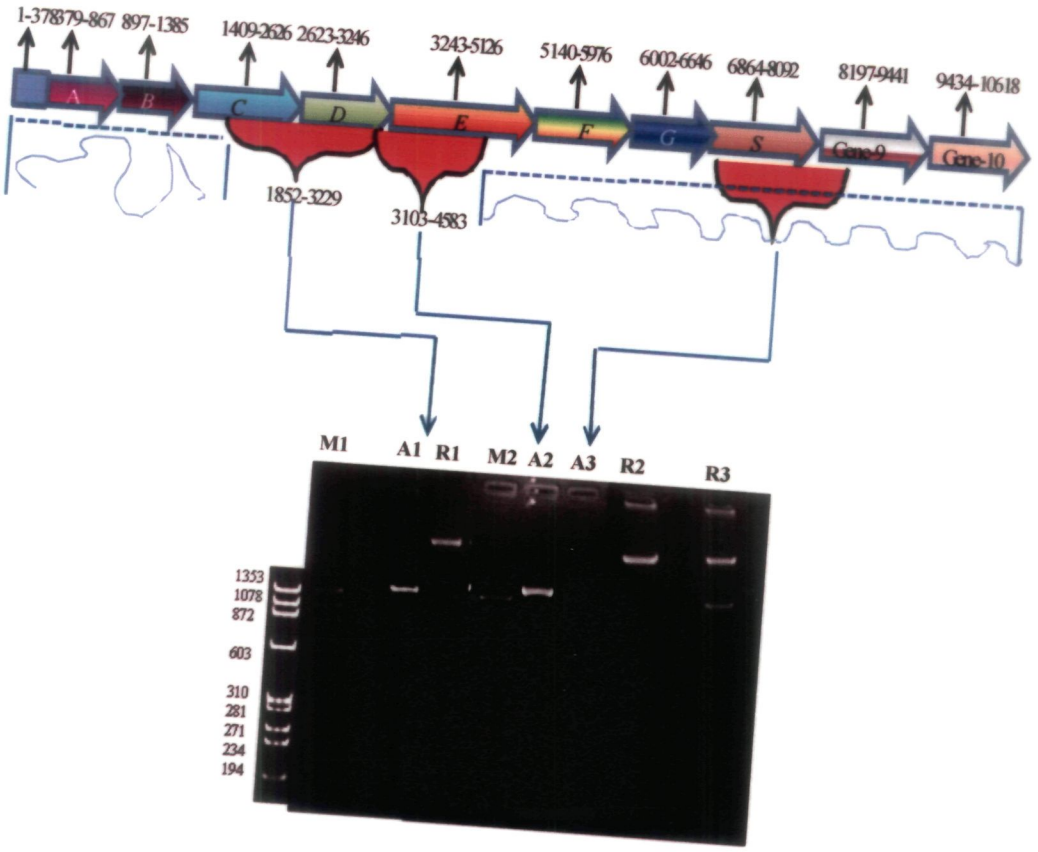


Fig.39. (A); *phz* operon organization of the SBJ1357 and genes targeted in this study; **(B)** Agarose gel electrophoresis of the amplified *phz*C,D,E&S genes from SBJ1357 genomic DNA

M1 and M2- □X174 *Hae* III DNA molecular weight marker.

A1- PCR amplified phz C&D genes (1.4 kb) of the SBJ1357 strain.

A2- PCR amplified phz S&9 genes (1.5 kb) of the SBJ1357 strain.

A3- PCR amplified phz D&E genes(1.48 kb) of the SBJ1357 strain.

R1, R2 and R3- *Eco*RI digested recombinant plasmids containing *phz* gene inserts.

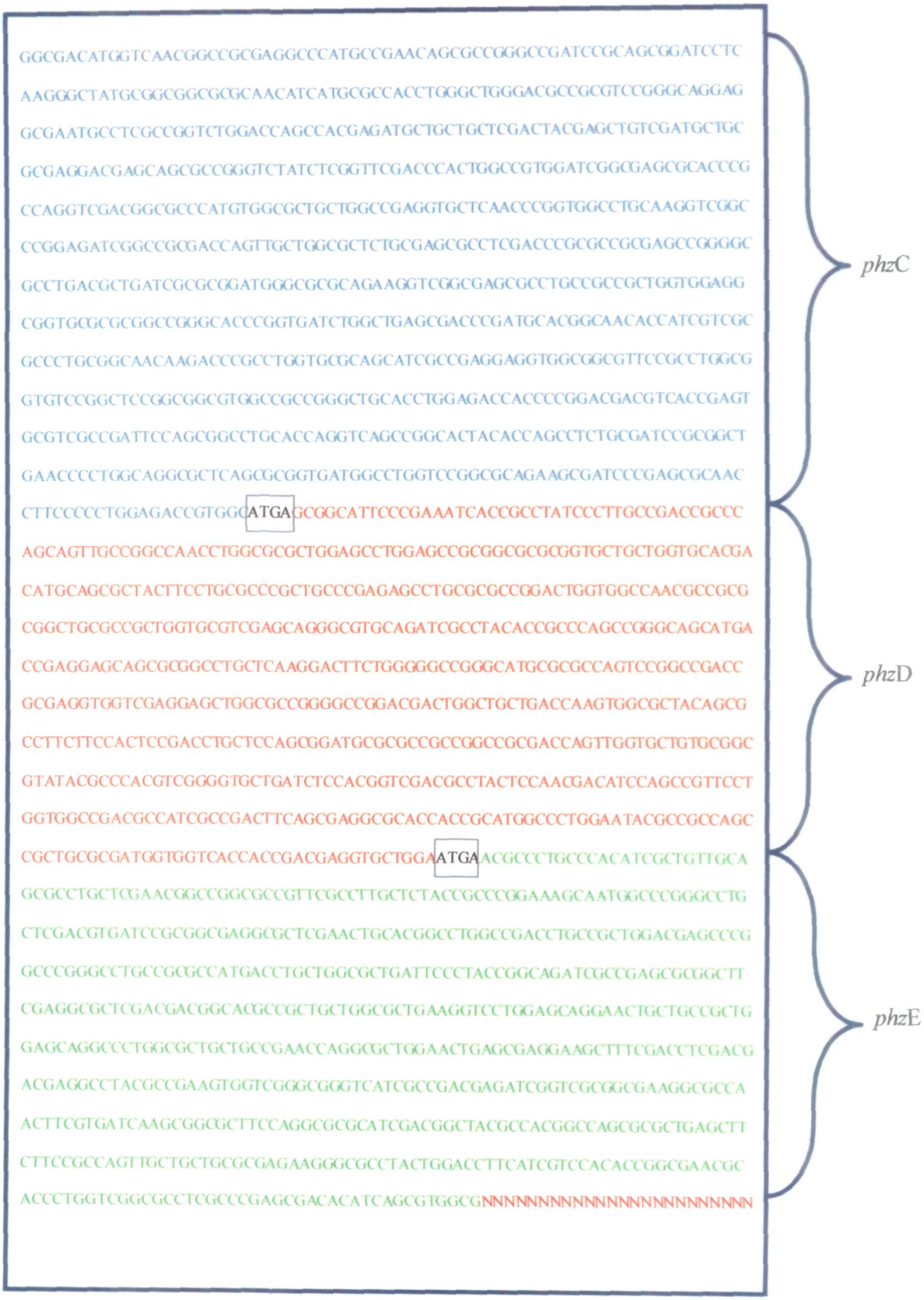


Fig.40. Nucleotide sequences of the *phz* operon genes of the strain SBJ1357 targeted in this study. The start and stop codons are indicated in the box.

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN *phz*E, F,G&S
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCTTGCCTTCG
GCCGCGTGGCGCGCCGAGATCGGATAGGCGACCAGGCGCGACCAGTGCTCGTCGTTG
GCGACGATCATGGTCTTGCCGTCGAGGAAGCGGTCGAACTCGGTGACGCCGCGCCAC
ATGGTGATCCCACCGTGGGACAGCGGCCCCTGGTCGGGATGCAGGTGCGCGCGGACC
GCCGTGTGGATGCCGTCGGCGCCGACCAGCACATCGGCACCGAGCGCCAGGGGCTTG
CCGTGTCCGTCGCGGGCGCCGATCAGTACGCGGCCGTCGCGCTCTTCGATGCGCTCCA *phzS*
CGCCGAGACCGGTGCGTACCGCCTGTTGGCCGAGACGCTCGCGCACCGCGGCGAGCA
GGATCATCTGCAGTTCGCCGCGATGGATCGAGTACTGCGGATAGGCGTTGCCGGCTT
CCACCCCGCGCGGCTCGGACCATACCGTGGCGCCGCTCTGGTCGATGTAGCGCAGCT
CGTGGGTGGGGATGGCGGTGGCCGCCAGAGCCGGGCCGAGACCCAGTTCGGCGAGG
GCCTCGACCGCCGCCGGCTGGATATTAATGCCGACGCCAAGGGGGCGTATCCCGCTG
CTGCTTTCCAGCAGCGTGACCTTGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN *phzS,Gene9&10*
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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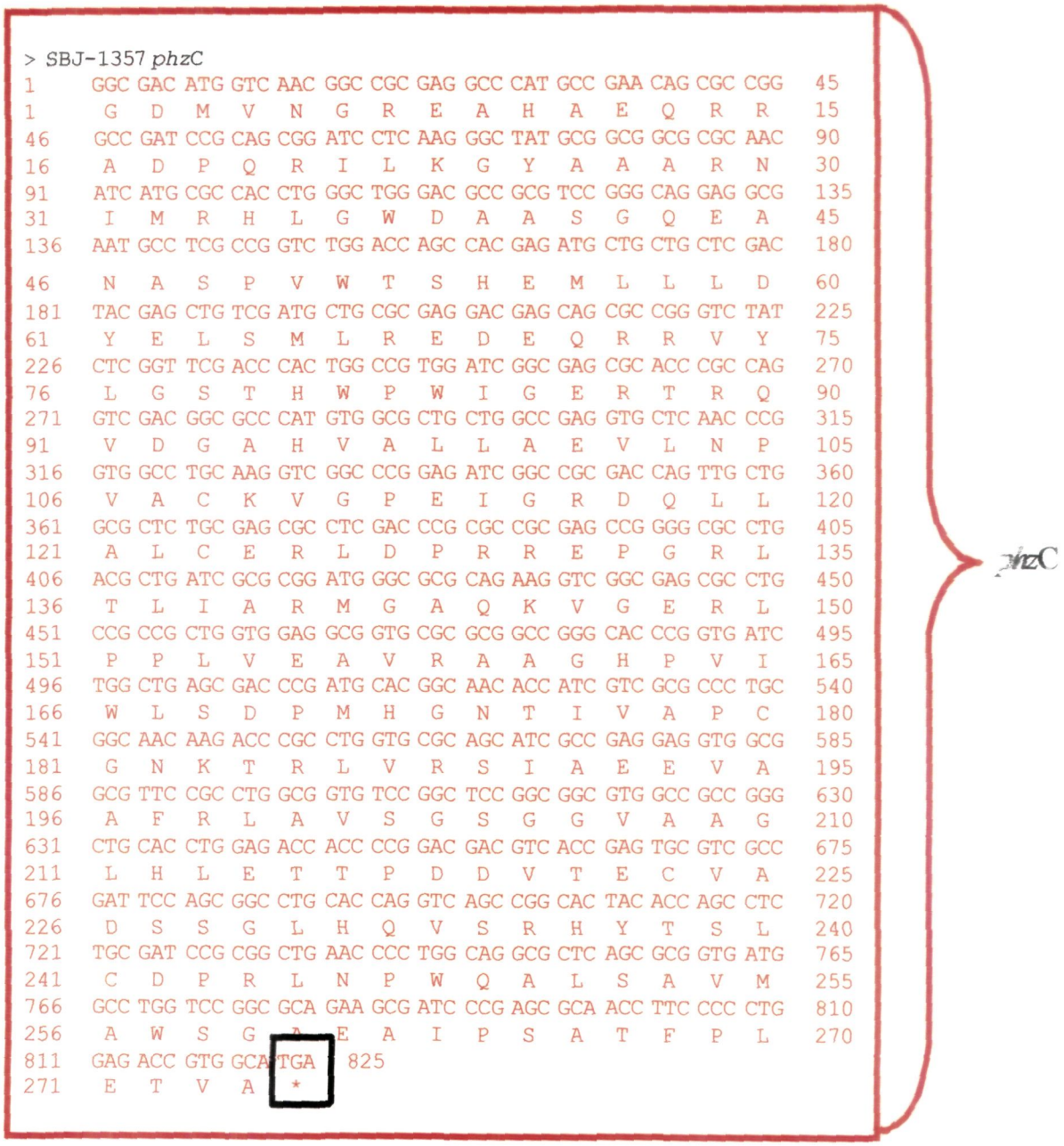


Fig.41. Nucleotide and translated amino acid sequences of the *phz*C gene of the strain SBJ1357. The stop codon of *phz*C is represented by box.

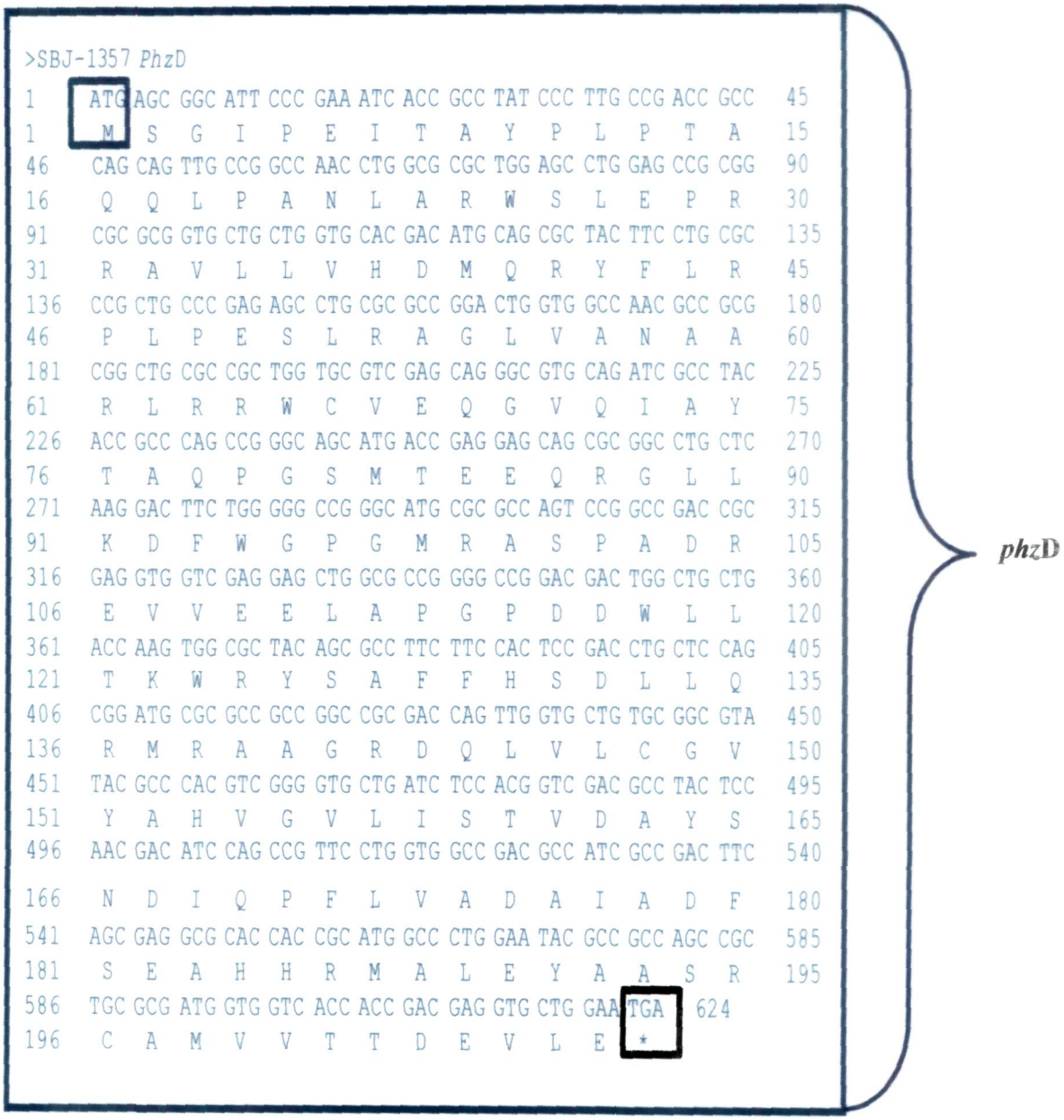


Fig.42. Nucleotide and translated amino acid sequences of the *phz*D gene of the strain SBJ1357. The stop and start codons of *phz*D is represented by box.

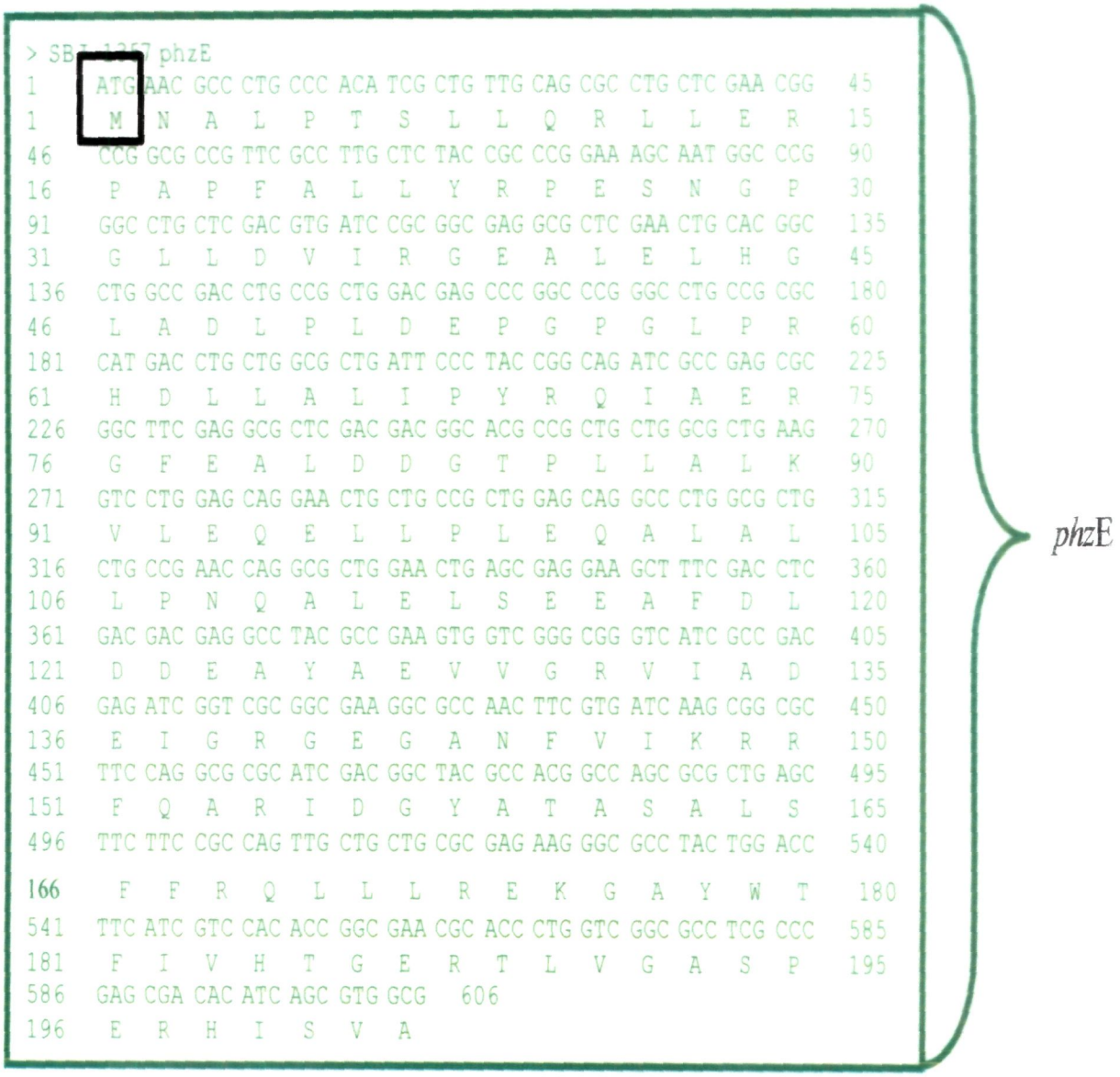


Fig.43. Nucleotide and translated amino acid sequences of the *phz*E gene of the strain SBJ1357. The start codon of *phz*E is represented by box.

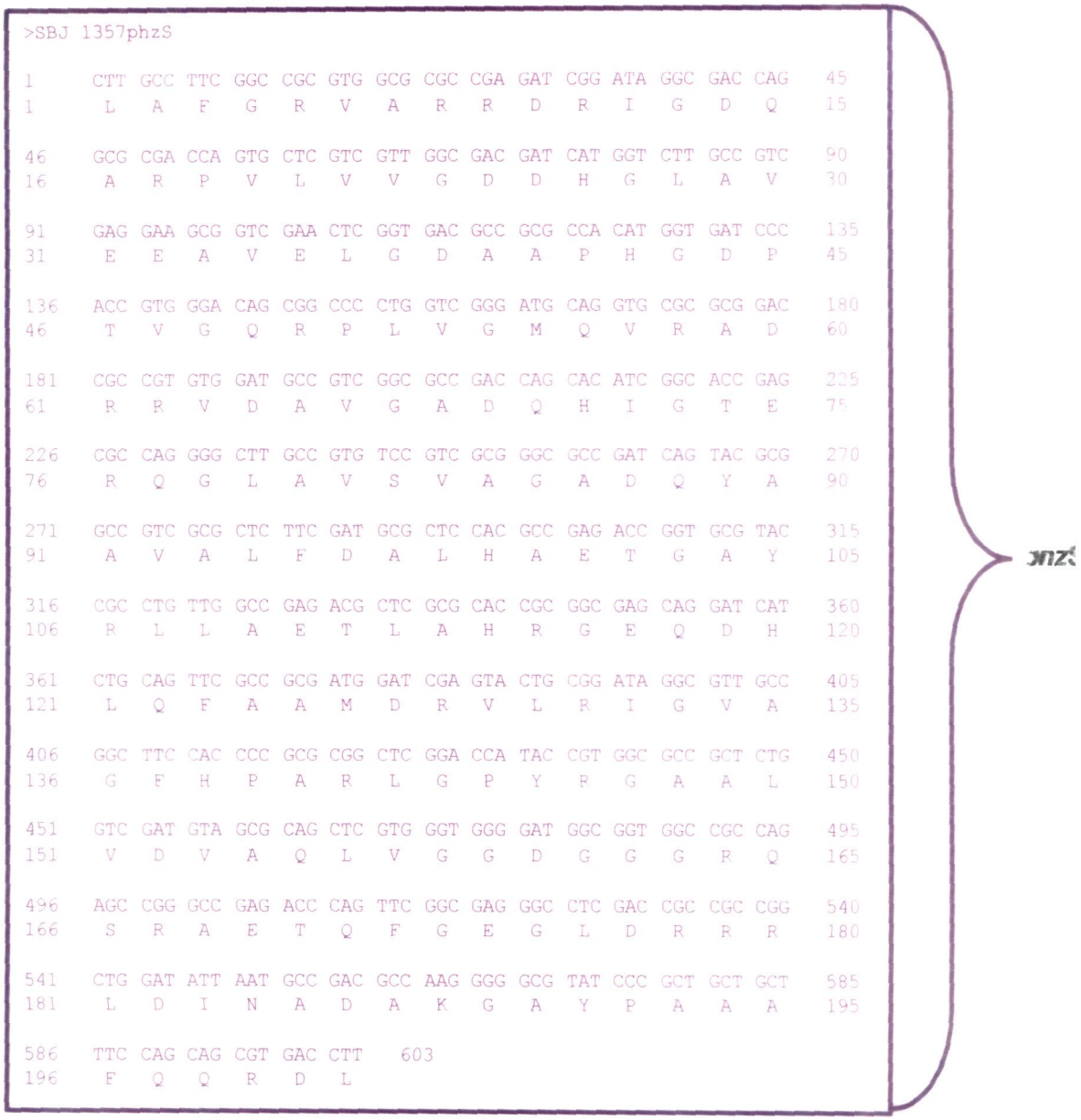


Fig.44. Nucleotide and translated amino acid sequences of the phzS gene of the strain SBJ1357.

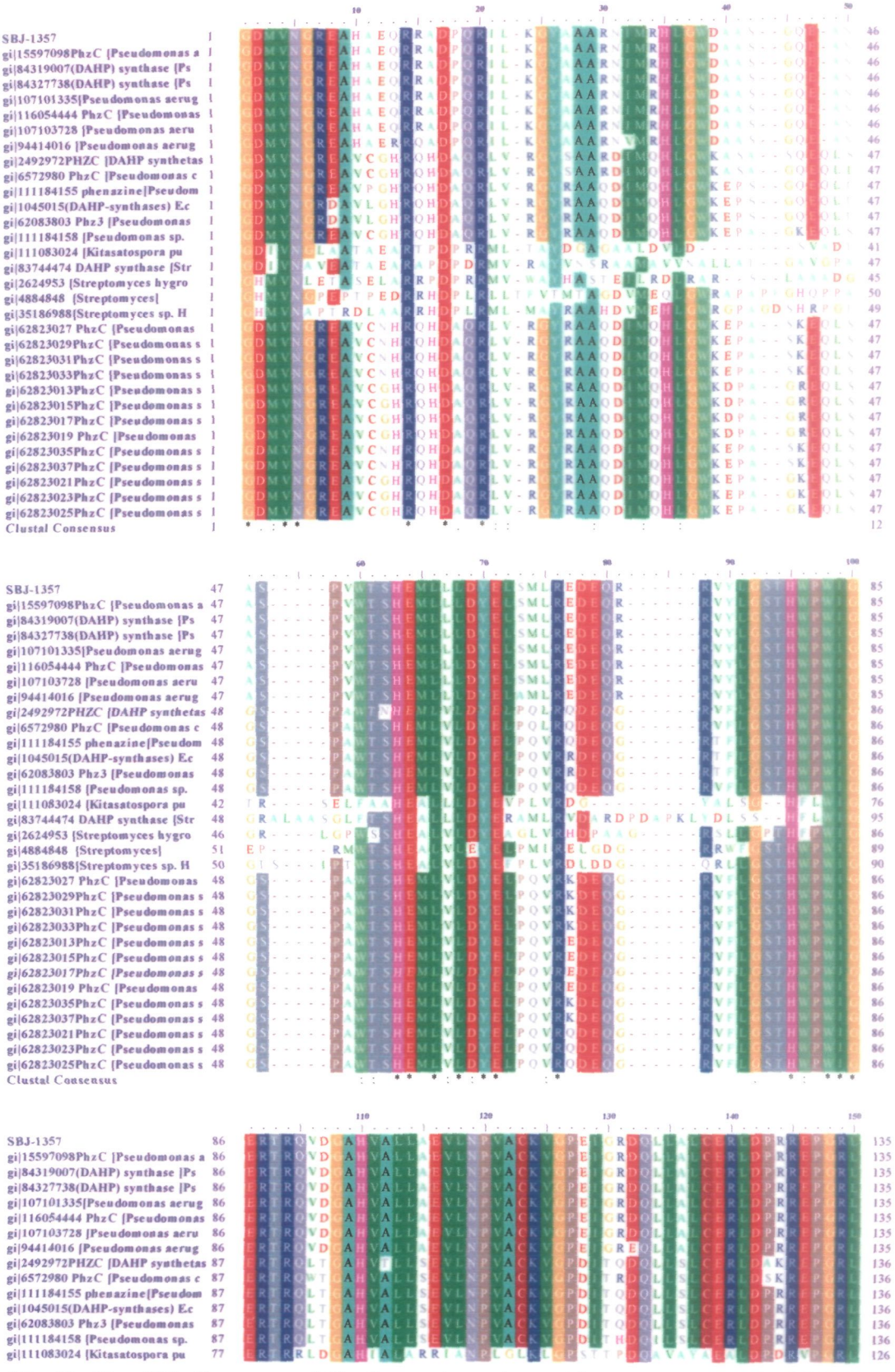
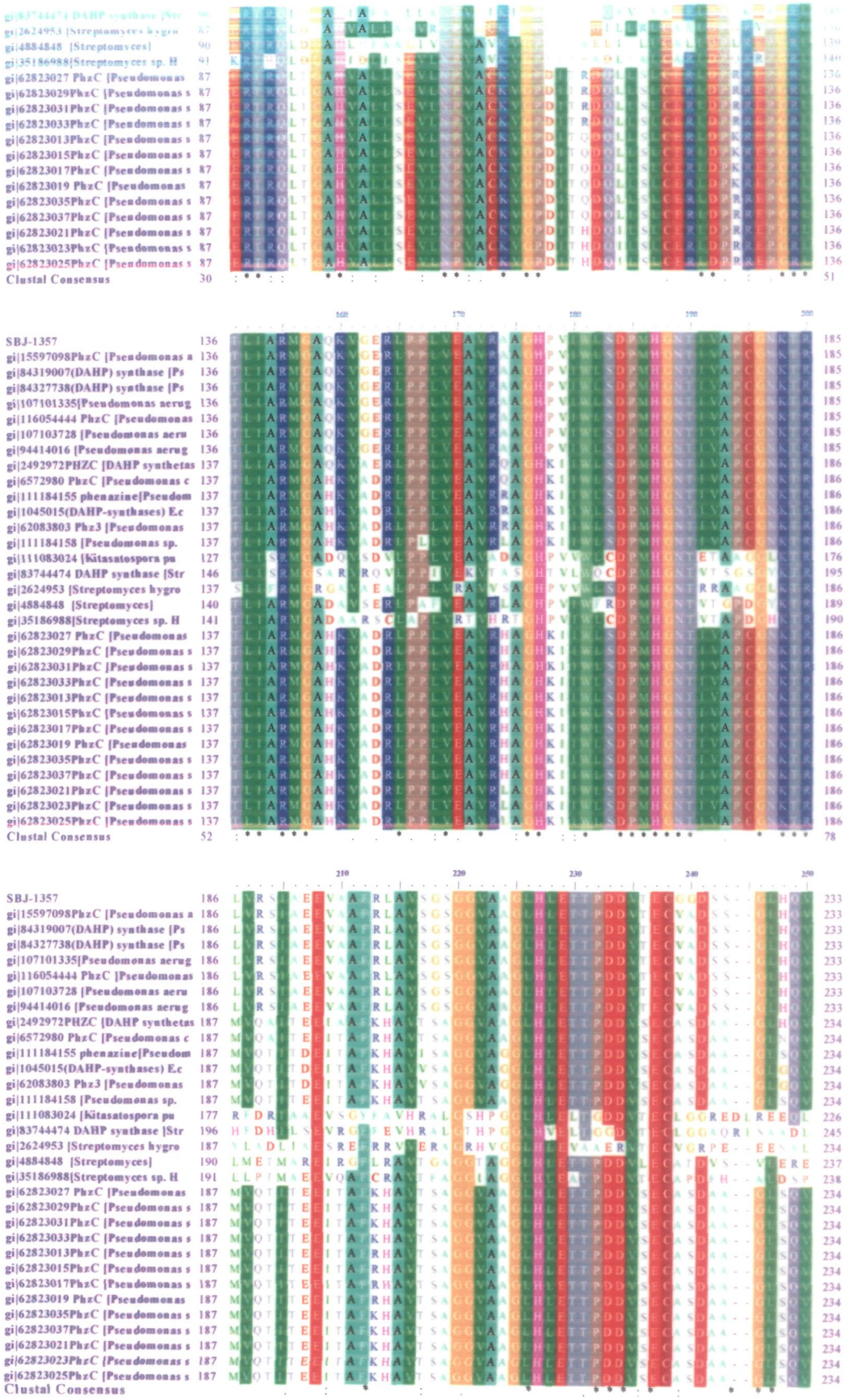
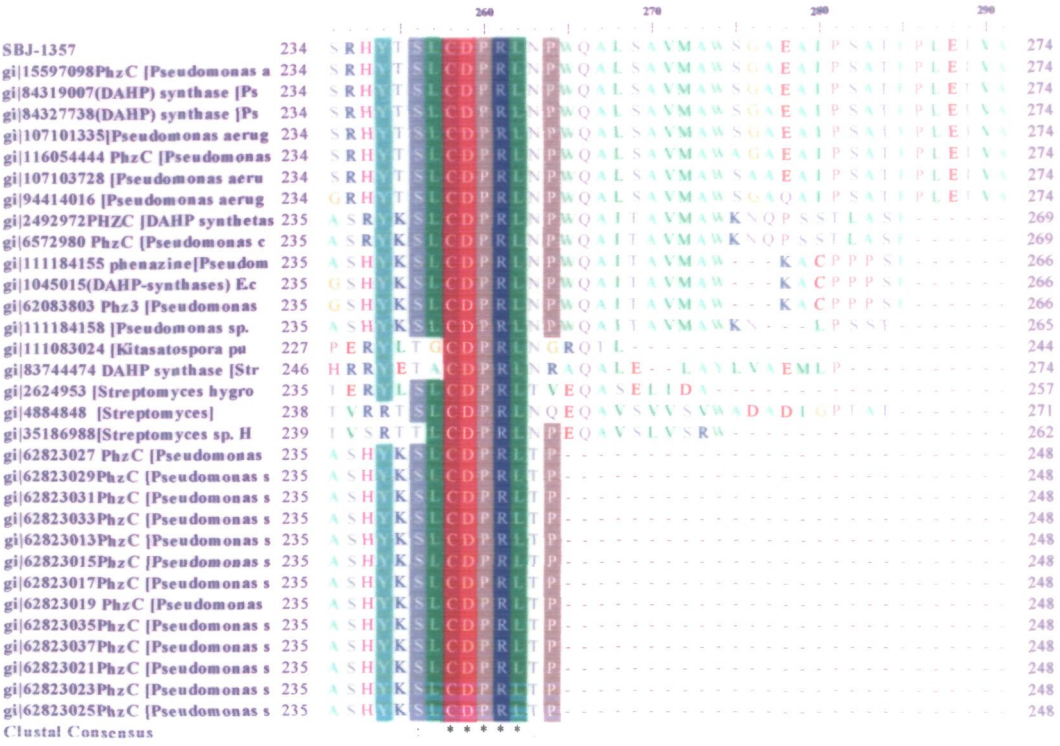
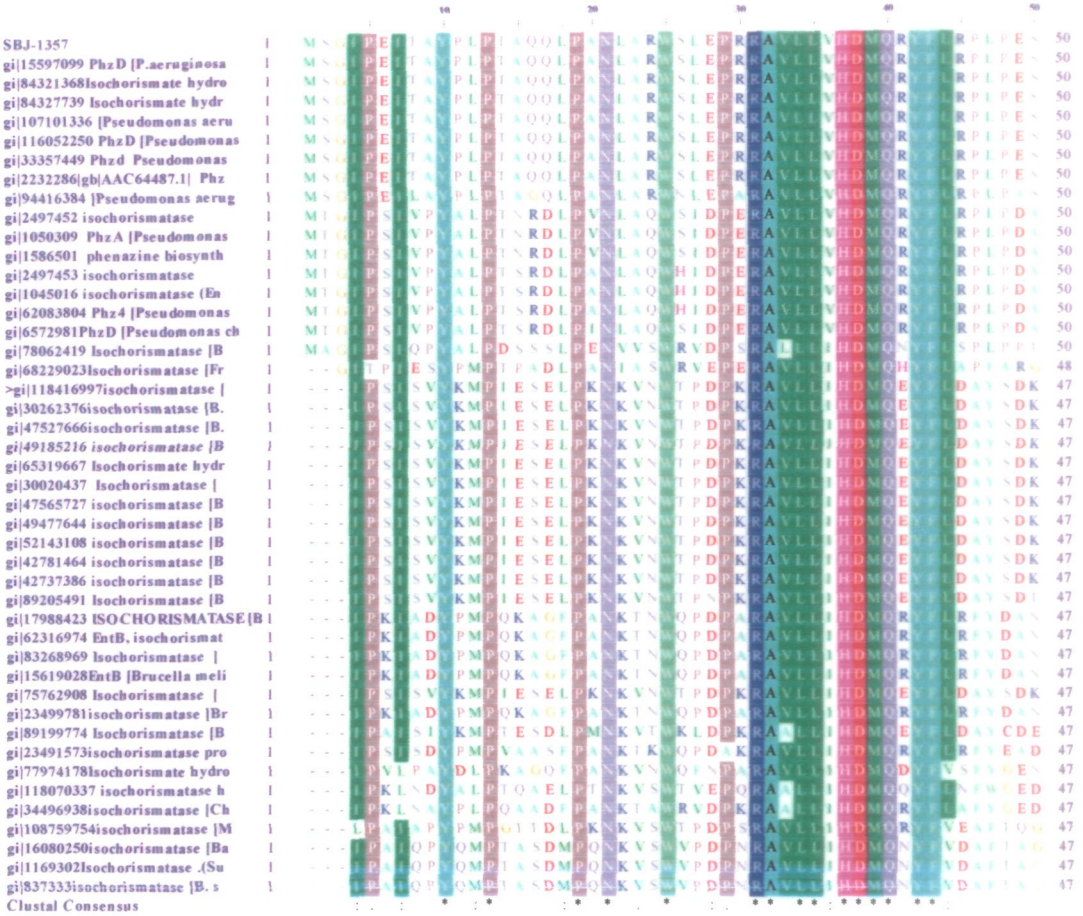
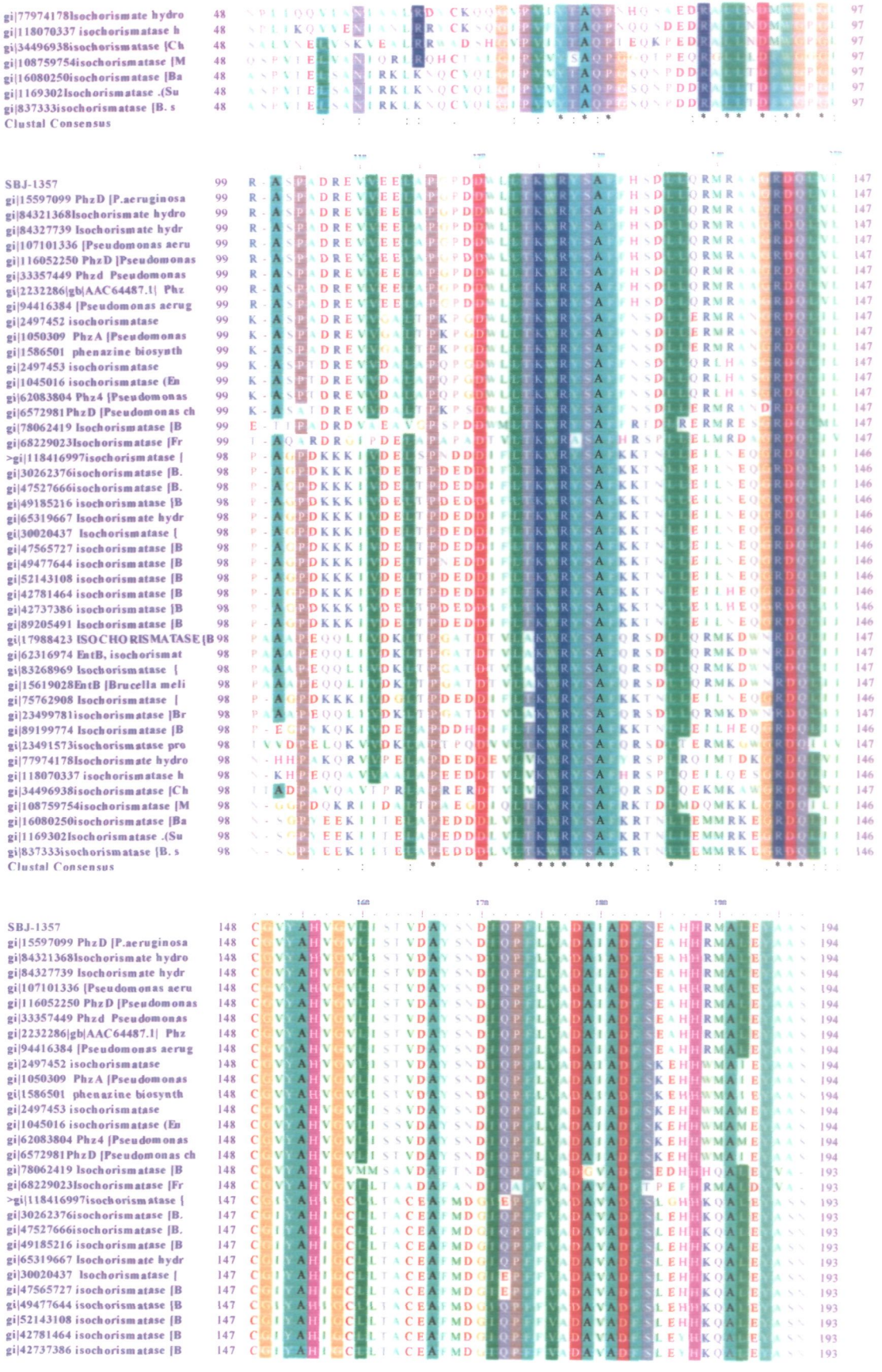


Fig.45. ClutalW alignment of PhzC protein amino acid sequence of strain SBJ1357 with reference strains of *Pseudomonas* spp. and other genera.




260
270
280
290
SBJ-1357 234 274
gi|15597098PhzC [Pseudomonas a 234 274
gi|84319007(DAHP) synthase [Ps 234 274
gi|84327738(DAHP) synthase [Ps 234 274
gi|107101335|Pseudomonas aerug 234 274
gi|116054444 PhzC [Pseudomonas 234 274
gi|107103728 [Pseudomonas aeru 234 274
gi|94414016 [Pseudomonas aerug 234 274
gi|2492972PHZC [DAHP synthetas 235 269
gi|6572980 PhzC [Pseudomonas c 235 269
gi|111184155 phenazine[Pseudom 235 266
gi|1045015(DAHP-synthases) E.c 235 266
gi|62083803 Phz3 [Pseudomonas 235 266
gi|111184158 [Pseudomonas sp. 235 265
gi|111083024 [Kitasatospora pu 227 244
gi|83744474 DAHP synthase [Str 246 274
gi|2624953 [Streptomyces hygro 235 257
gi|4884848 [Streptomyces] 238 271
gi|35186988[Streptomyces sp. H 239 262
gi|62823027 PhzC [Pseudomonas 235 248
gi|62823029PhzC [Pseudomonas s 235 248
gi|62823031PhzC [Pseudomonas s 235 248
gi|62823033PhzC [Pseudomonas s 235 248
gi|62823013PhzC [Pseudomonas s 235 248
gi|62823015PhzC [Pseudomonas s 235 248
gi|62823017PhzC [Pseudomonas s 235 248
gi|62823019 PhzC [Pseudomonas 235 248
gi|62823035PhzC [Pseudomonas s 235 248
gi|62823037PhzC [Pseudomonas s 235 248
gi|62823021PhzC [Pseudomonas s 235 248
gi|62823023PhzC [Pseudomonas s 235 248
gi|62823025PhzC [Pseudomonas s 235 248
Clustal Consensus


SBJ-1357
gi|15597099 PhzD [P.aeruginosa
gi|84321368Isochorismate hydro
gi|84327739 Isochorismate hydr
gi|107101336 [Pseudomonas aeru
gi|116052250 PhzD [Pseudomonas
gi|33357449 Phzd Pseudomonas
gi|2232286|gb|AAC64487.1| Phz
gi|94416384 [Pseudomonas aerug
gi|2497452 isochorismatase
gi|1050309 PhzA [Pseudomonas
gi|1586501 phenazine biosynth
gi|2497453 isochorismatase
gi|1045016 isochorismatase [En
gi|62083804 Phz4 [Pseudomonas
gi|6572981PhzD [Pseudomonas ch
gi|78062419 Isochorismatase [B
gi|68229023Isochorismatase [Fr
>gi|118416997isochorismatase [
gi|30262376isochorismatase [B.
gi|47527666isochorismatase [B.
gi|49185216 isochorismatase [B
gi|65319667 Isochorismate hydr
gi|30020437 Isochorismatase [
gi|47565727 isochorismatase [B
gi|49477644 isochorismatase [B
gi|52143108 isochorismatase [B
gi|42781464 isochorismatase [B
gi|42737386 isochorismatase [B
gi|89205491 Isochorismatase [B
gi|17988423 ISOCHORISMATASE [B
gi|62316974 EntB, isochorismat
gi|83268969 Isochorismatase [
gi|15619028EntB [Brucella meli
gi|75762908 Isochorismatase [
gi|23499781isochorismatase [Br
gi|89199774 Isochorismatase [B
gi|23491573isochorismatase pro
gi|77974178Isochorismate hydro
gi|118070337 isochorismatase h
gi|34496938isochorismatase [Ch
gi|108759754isochorismatase [M
gi|16080250isochorismatase [Ba
gi|1169302Isochorismatase /Su
gi|837333isochorismatase [B. s
Clustal Consensus



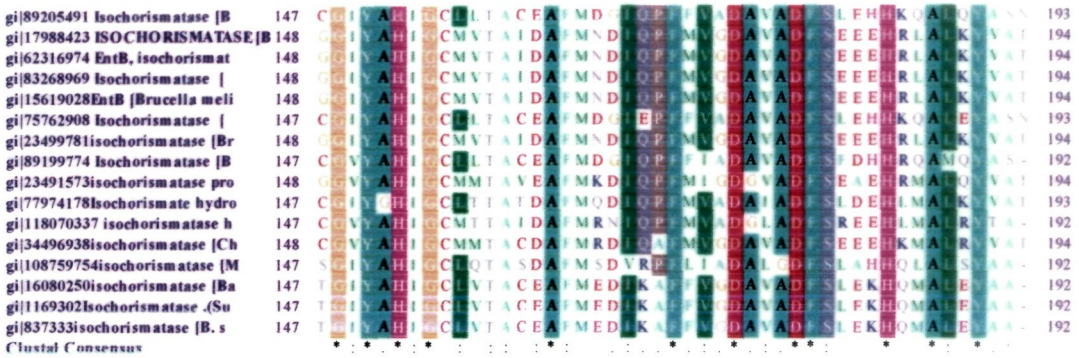


Fig.46. ClutalW alignment of PhzD protein amino acid sequence of strain SBJ1357 with reference strains of *Pseudomonas* spp. and other genera.

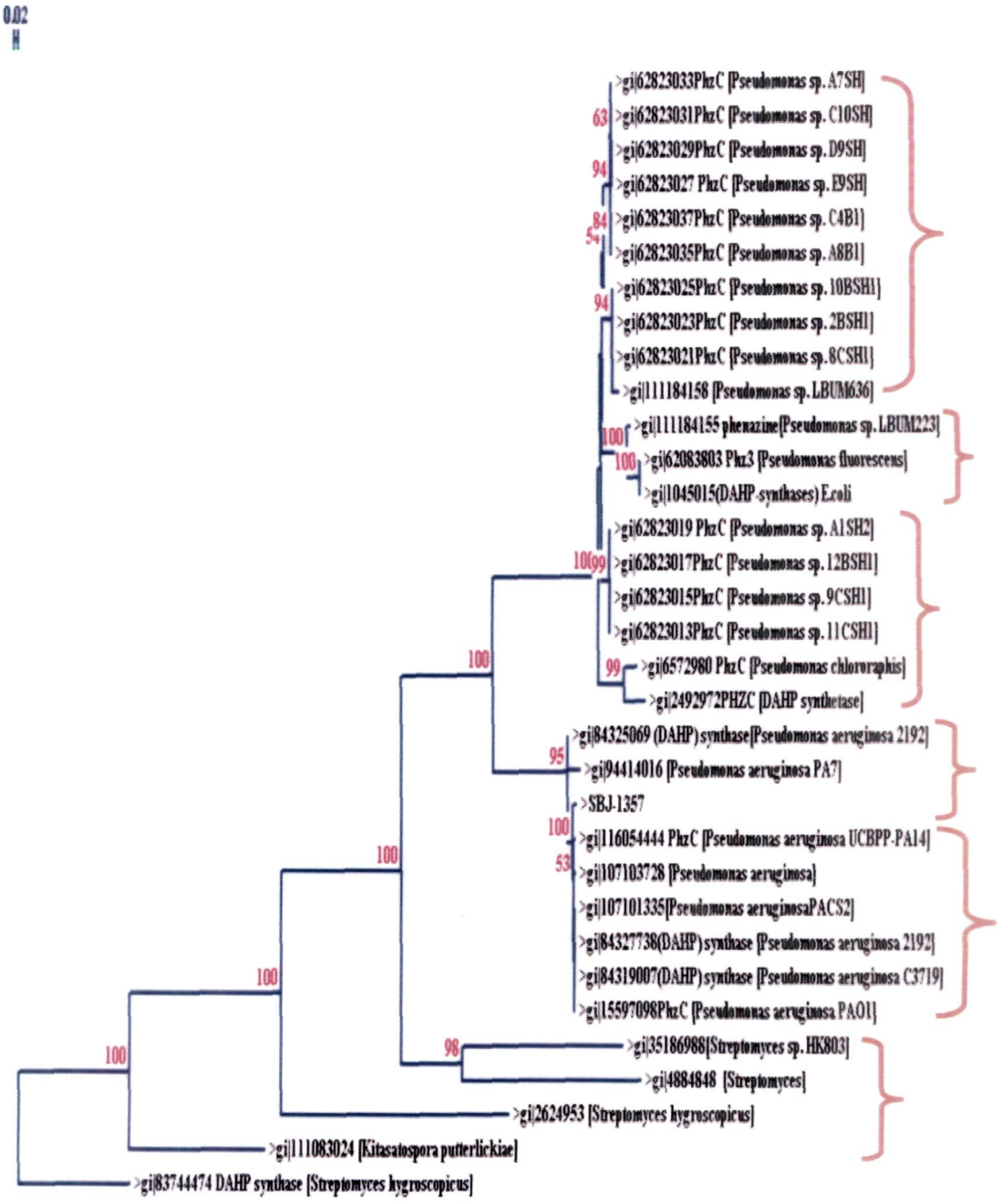


Fig.47. Phylogenetic relationship based on PhzC amino acid sequence of strain SBJ1357 and reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40 % or greater are indicated on the tree. The scale bar indicates the numbers of amino acid substitutions per site.

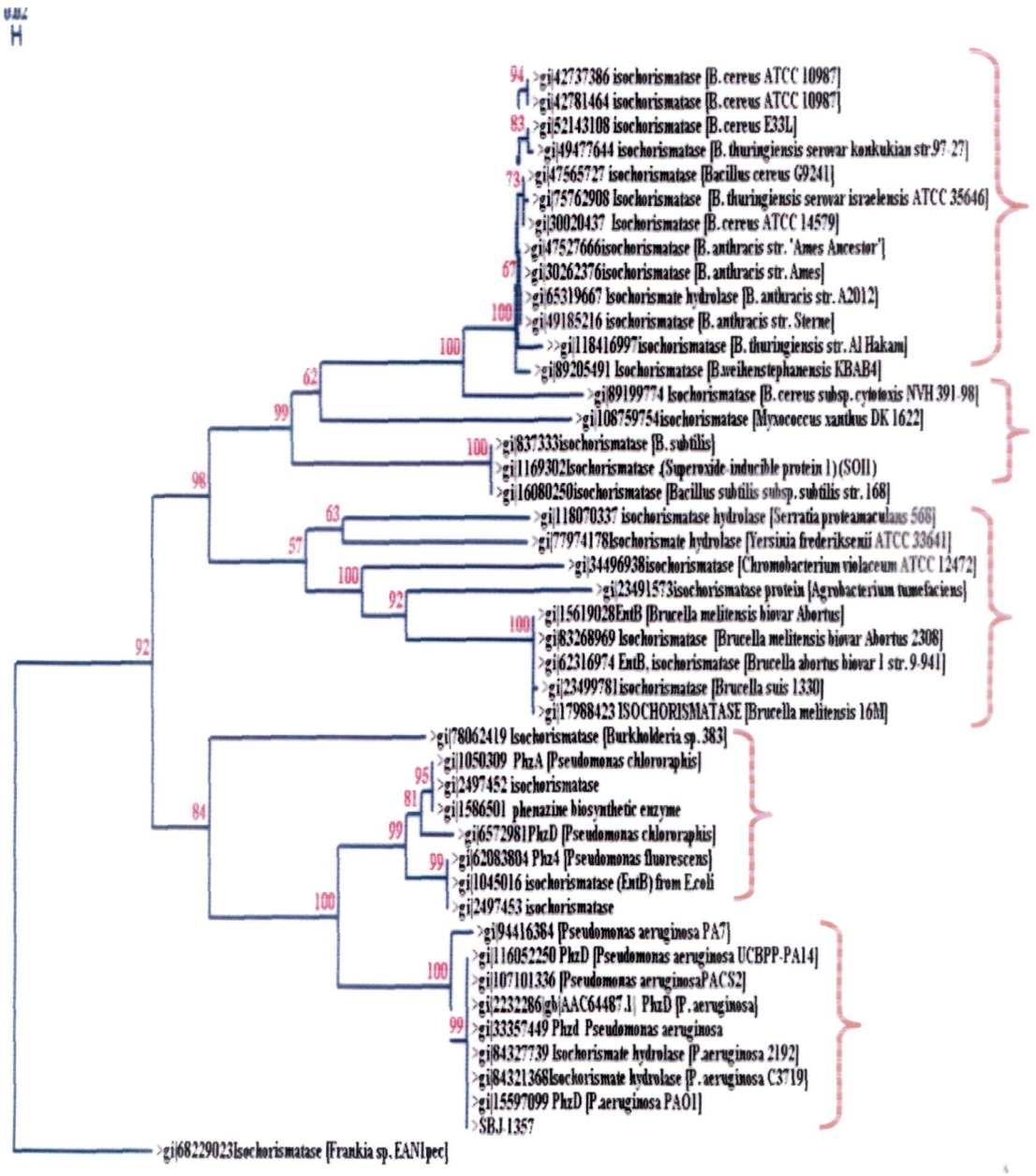


Fig.48. Phylogenetic relationship based on PhzD amino acid sequences of strain SBJ1357 and reference strains. The distance method of neighbor-joining was used to construct the tree topology. All bootstrap values of 40 % or greater are indicated on the tree. The scale bar indicates the numbers of amino acid substitutions per site.

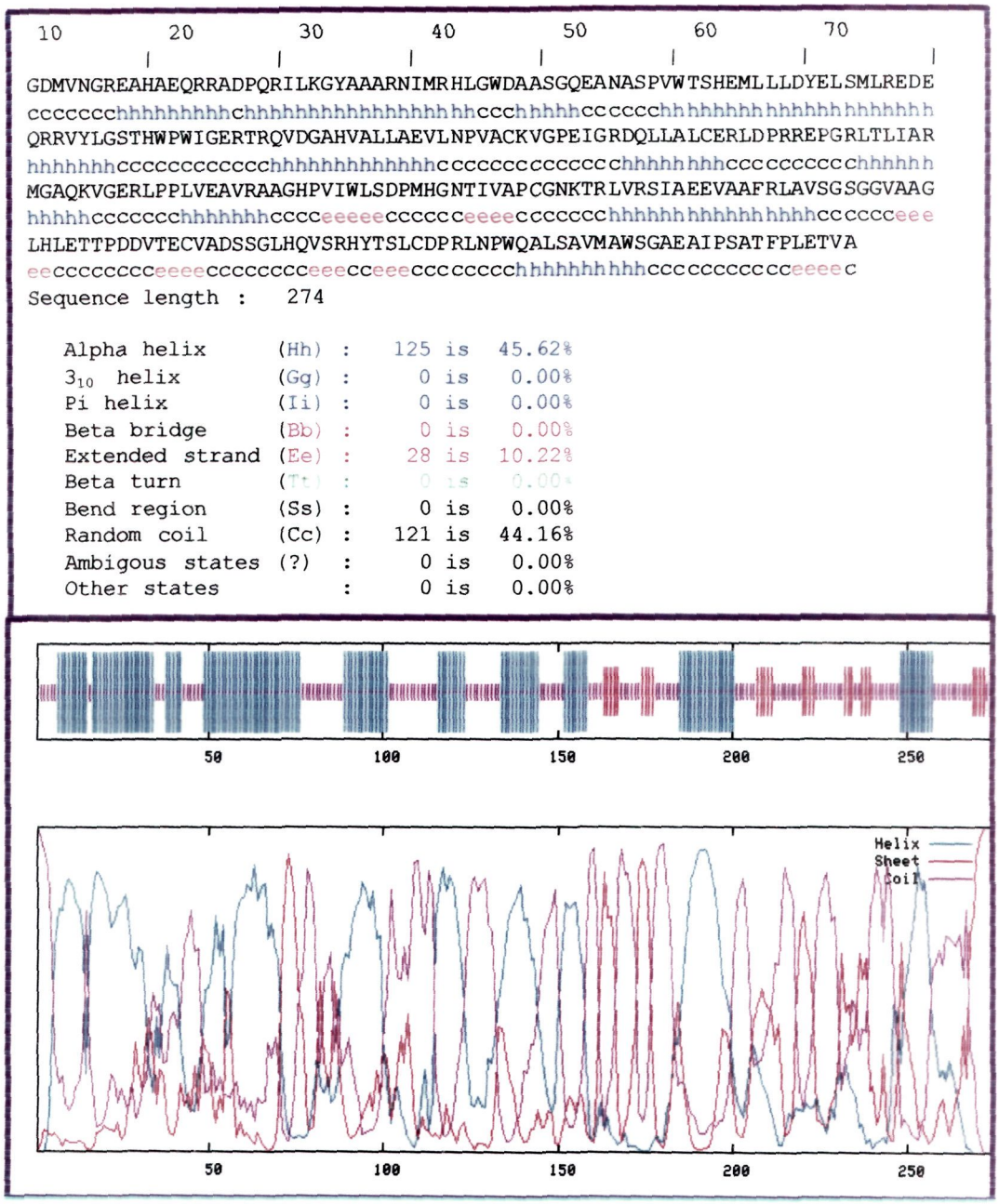


Fig.49. Secondary structure of the PhzC protein of strain SBJ1357.

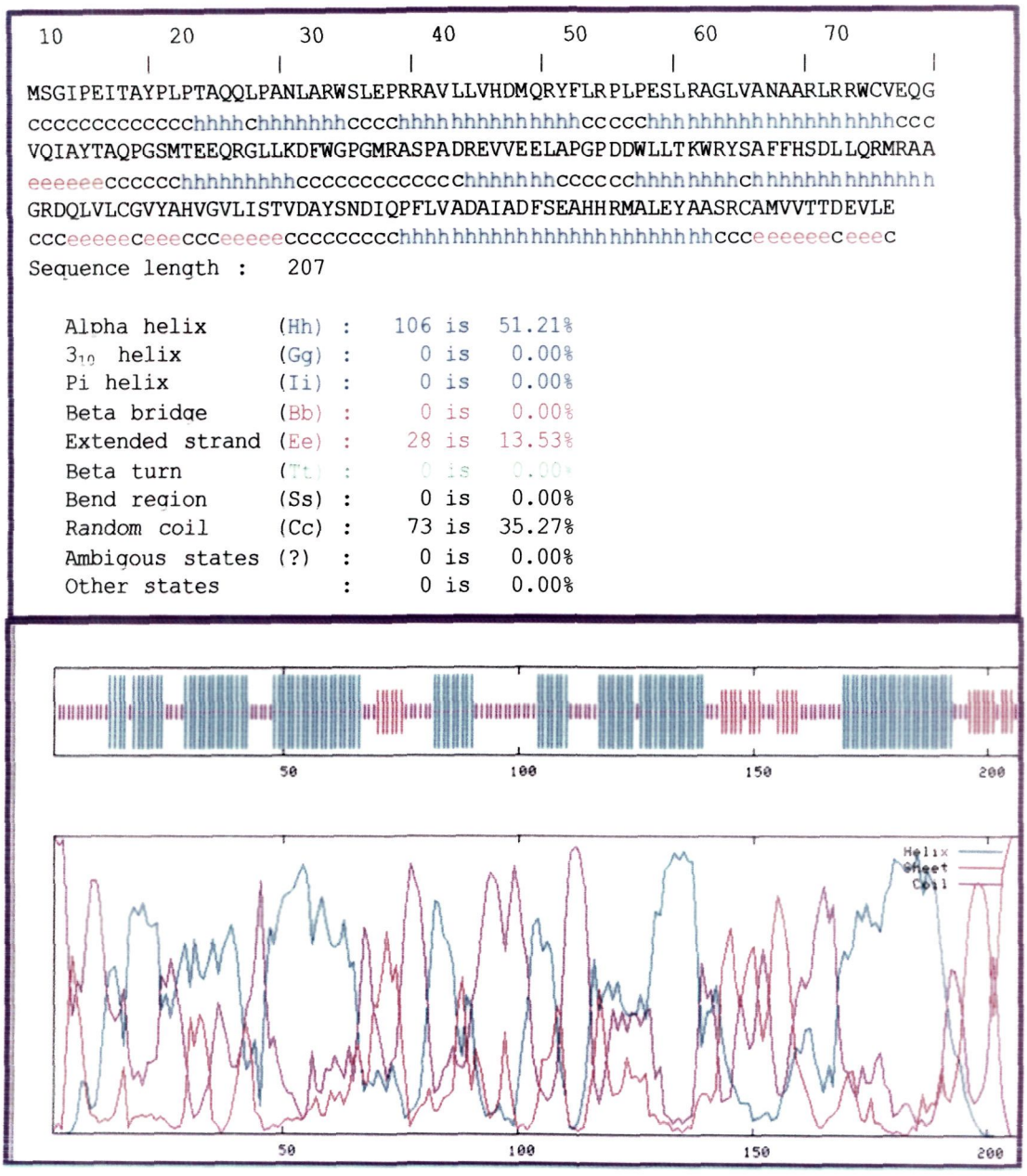


Fig.50. Secondary structure of the PhzD protein of strain SBJ1357.

Besides, the conserved domains search of PhzC &D proteins revealed the similarity with the enzymatic (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) structure from *Mycobacterium tuberculosis* [gi 82408028PDB 2B7O (MMDB 35706)] (Figs.53 and 54). In contrast to PhzC protein, PhzD protein of the strain SBJ1357 revealed the similarity with the enzymatic (2,3 dihydro2,3-dihyroxybenzoate synthase) structure from *Pseudomonas aeruginosa* PAO1 (Figs.55 and 56). The 3D structure based fold recognition study of the PhzD protein exhibited 13 % to 10 % identity and 100 % estimated precision with different Isochorismatase-like hydrolases. The Isochorismatase-like hydrolases of strain SBJ1357 also showed the structural homologies to eukaryotic hydrolases (yeast, *caenorhabditis elegans*) (Table 5). 3D structure of PhzD of SBJ1357 was constructed by homology modeling using X-ray crystallographic structure of the PDB: 1nf9 as a template. The 3D Model for PhzD of strain SBJ1357 was constructed and verified. The model has 8888.06 kcal/mol energy and the Ramachandran plot qualities do not show the amount (%) of residues belonging to the disallowed region of the plot. The rms deviations (A°) from template backbone 0.401041 values indicated the overall deviation of the 3D structure from the template (Figs. 55 to 58). The 3D structural analysis of PhzC, E &S was not performed due to the partial sequencing.

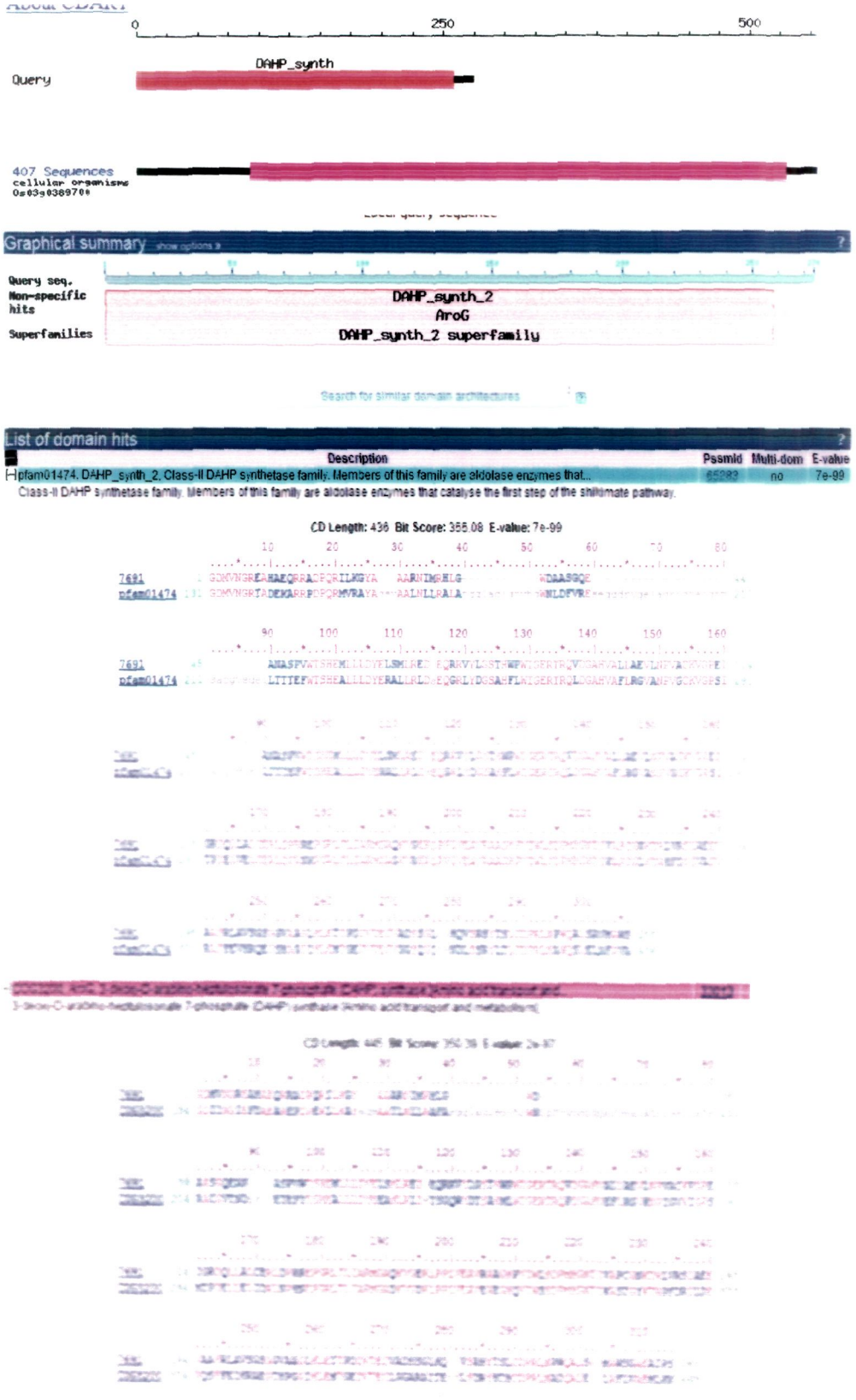


Fig.51. CARDT showing the presence of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase conserved domain in the strain SBJ 1357 *phz*C gene encoding PhzC protein. The PhzC of the strain SBJ1357 exhibited the homology and alignment with super family DAPH synthase.

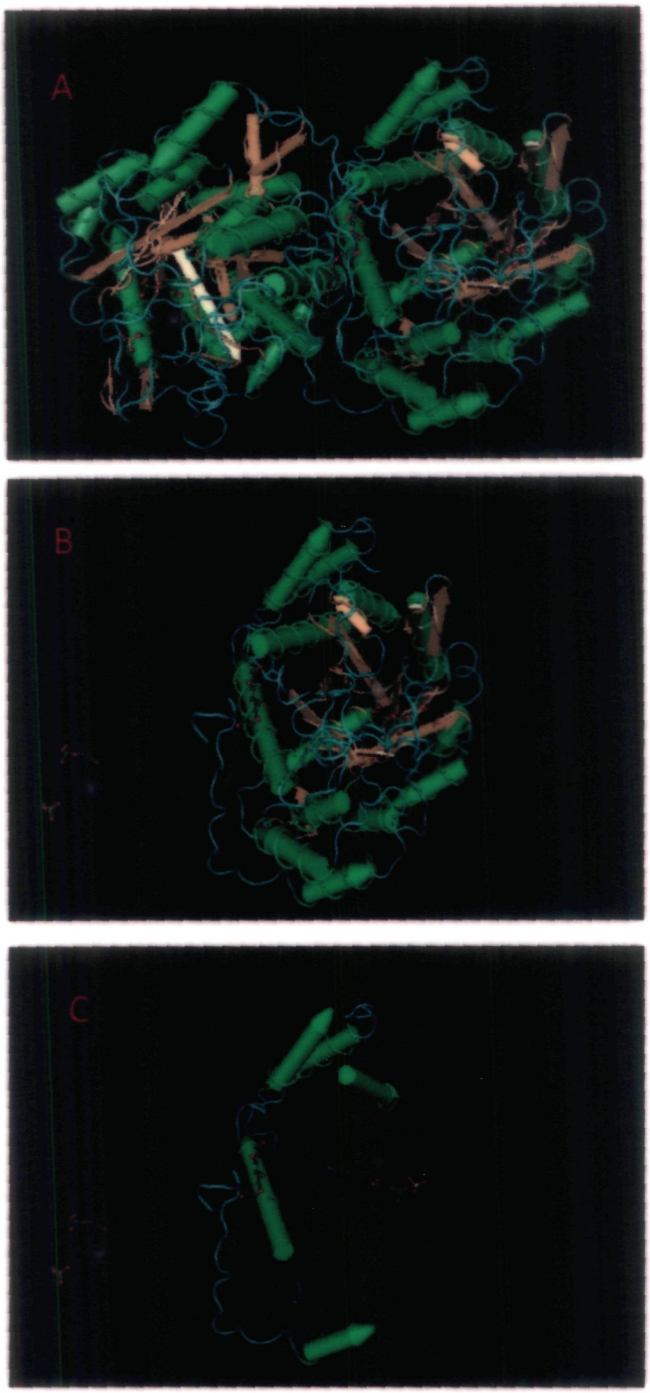


Fig.52. Presence of 3-deoxy-D-arabino-heptulosonate 7- Phosphate synthase conserved domain in the strain SBJ1357 *phz*C gene encoding PhzC protein. The Enzymatic domain of the SBJ 1357 exhibited the highest homology toward the structure of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Mycobacterium tuberculosis* [gi 82408028PDB 2B7O (MMDB 35706)].

(A) Complete domains present in the 3-Deoxy-D-Arabino-Heptulosonate 7- Phosphate Synthase.

(B) Showing the all aligned domain of the strain SBJ1357 PhzC protein.

(C) Showing the unaligned residues in the 3-deoxy-D-arabino-heptulosonate 7-phosphate

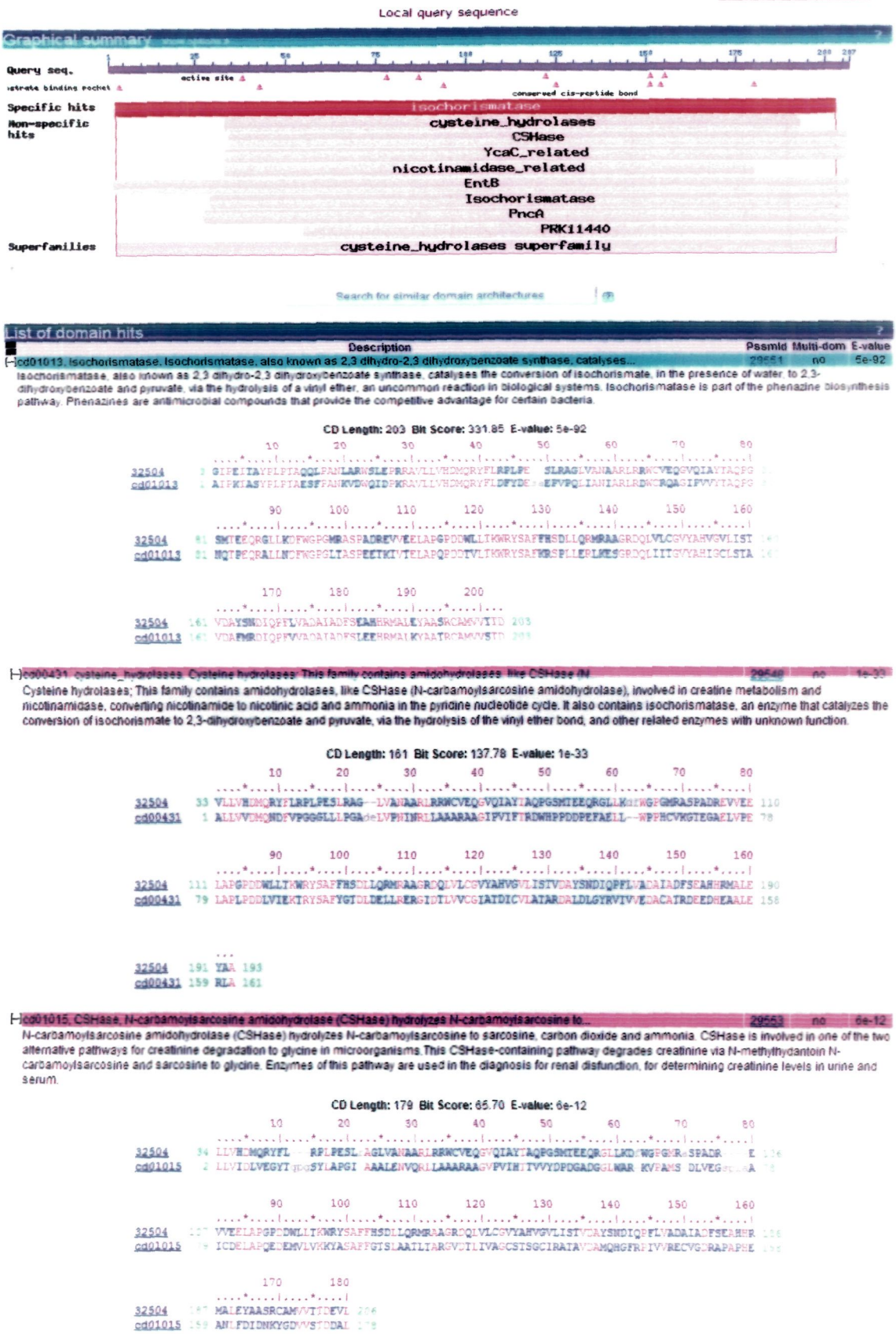


Fig.53. CARDT showing the presence of 2,3 dihydro2,3-dihyroxybenzoate synthase conserved domain in the strain SBJ 1357 *phz*D gene encoding PhzD protein. The PhzD of the strain SBJ1357 exhibited the homology and alignment with super family Cysteine hydrolases.

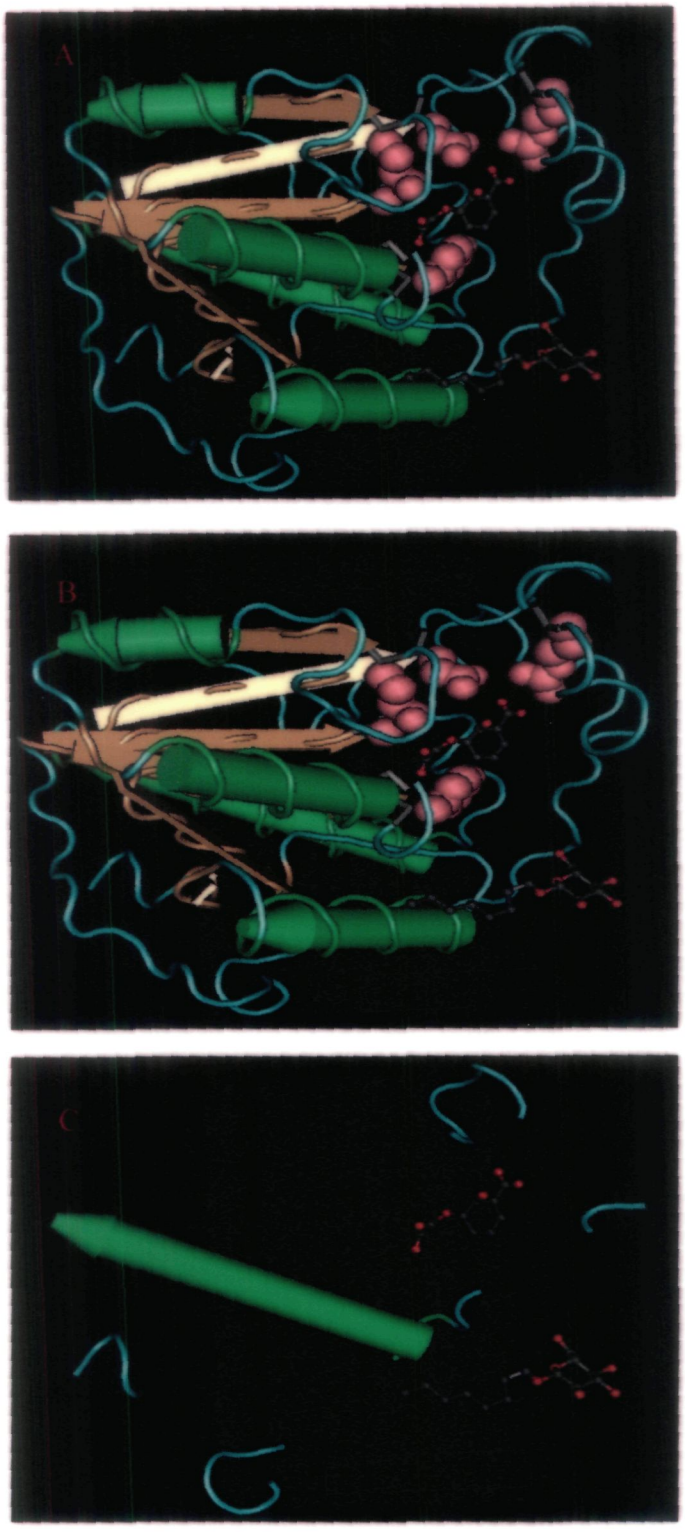


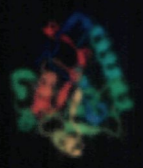
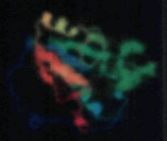
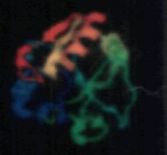
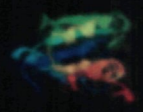
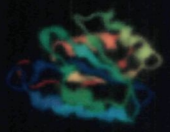
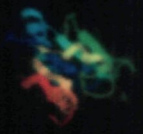
Fig.54. Presence of 2, 3-dihydro2,3-dihyroxybenzoate synthase conserved domain in the strain SBJ 1357 *phz*D gene encoding PhzD protein. The enzymatic domain of the SBJ 1357 exhibited the highest homology toward the structure of 2,3 dihydro2,3-dihyroxybenzoate synthase from *Pseudomonas aeruginosa* PAO1 [gi 82408028PDB 2B7O (MMDB 35706)].

(A) Complete domains present in the 2,3 dihydro2,3-dihyroxybenzoate synthase.

(B) Showing the all aligned domain of the strain SBJ1357 PhzC protein.

(C) Showing the unaligned residues in the 2,3 dihydro2,3-dihyroxybenzoate synthase domain.

Table.5. 3D-fold recognition ranking and alignments of the PhzD protein of strain SBJ1357.

SCOP Code	Estimated Precision	Fold/PDB descriptor	Family	Model
d1nf9a (length 207) 100% i.d.	100 %	Isochorismatase-like hydrolases	Isochorismatase-like hydrolases	
c2fq1A (length 287) 46% i.d.	100 %	Hydrolase	Non-ribosomal peptide2 synthetase entb containing Isochorismate lyase and aryl-3 carrier protein domains	
c2h0rA (length 216) 15% i.d.	100 %	Hydrolase	Structure of the yeast nicotinamidase pnc1p	
d1yaca (length 204) 17% i.d.	100 %	Isochorismatase-like hydrolases	Isochorismatase-like hydrolases	
c2b34G (length 199) 15% i.d.	100 %	Hydrolase	Structure of mar1 ribonuclease from *caenorhabditis elegans*	
c1yzvA (length 204) 13% i.d.	100 %	Structural genomics, unknown function	hypothetical protein from *trypanosoma cruzi*	

Title	Crystal Structure of PhzD protein from *Pseudomonas aeruginosa*
Authors	Parsons, F., Calabrese, K., Eisenstein, E., Ladner, J.E.
Primary citation	Parsons, J.F., Calabrese, K., Eisenstein, E., Ladner, J.E. (2003) Structure and mechanism of *Pseudomonas aeruginosa* PhzD, an isochorismatase from the phenazine biosynthetic pathway Biochemistry 42: 5684-5693
History	Deposition 2002-12-13 Release 2003-06-17
Experimental method	X-RAY DIFFRACTION Data
PDB code	1NF9

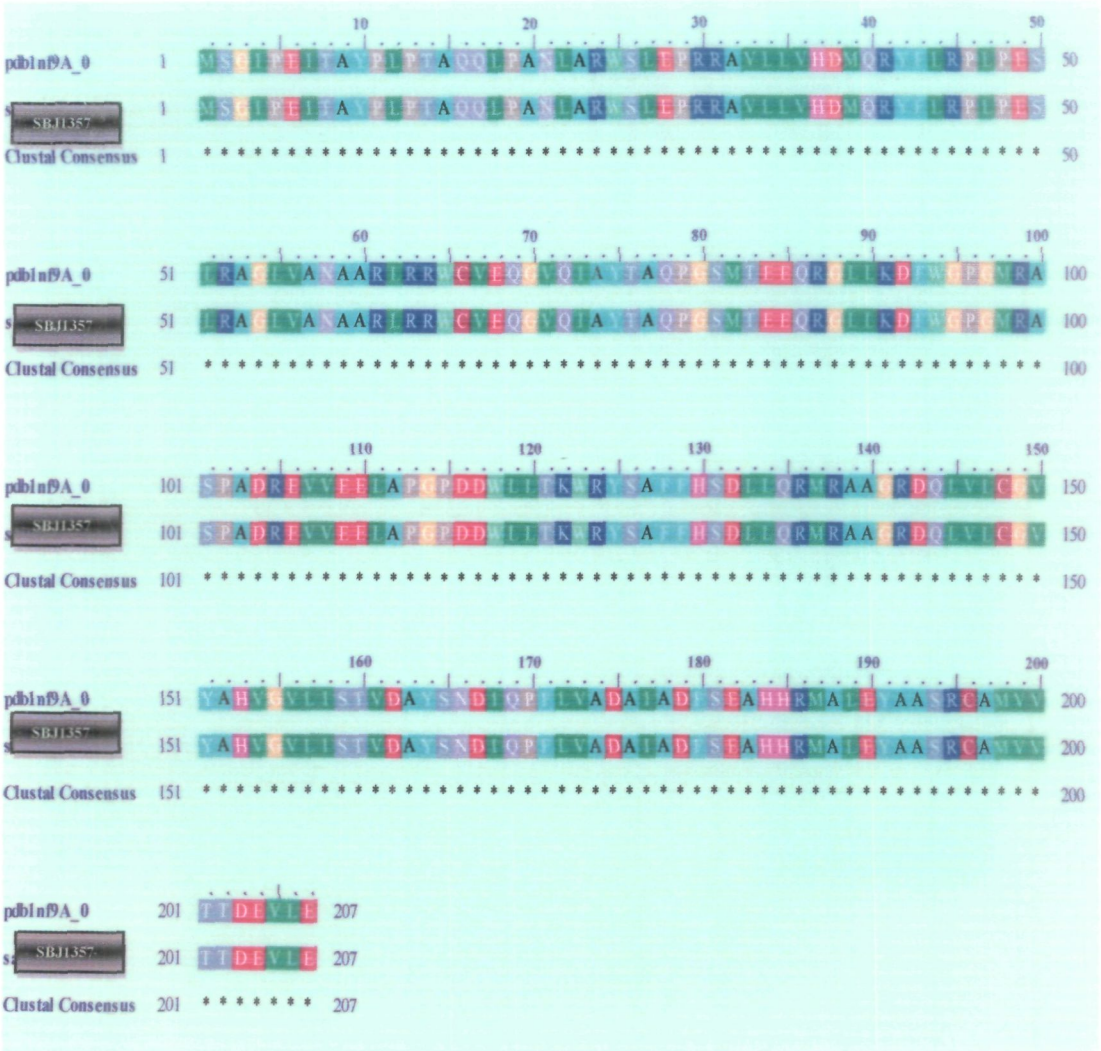


Fig.55. Summary of the template X-ray crystallographic structure used for the comparative modeling PhzD protein encoded by *phzD* gene of strain SBJ1357. **(B);** Alignment of the amino acid sequences of template and the PhzD protein encoded by *phzD* gene of strain SBJ1357.

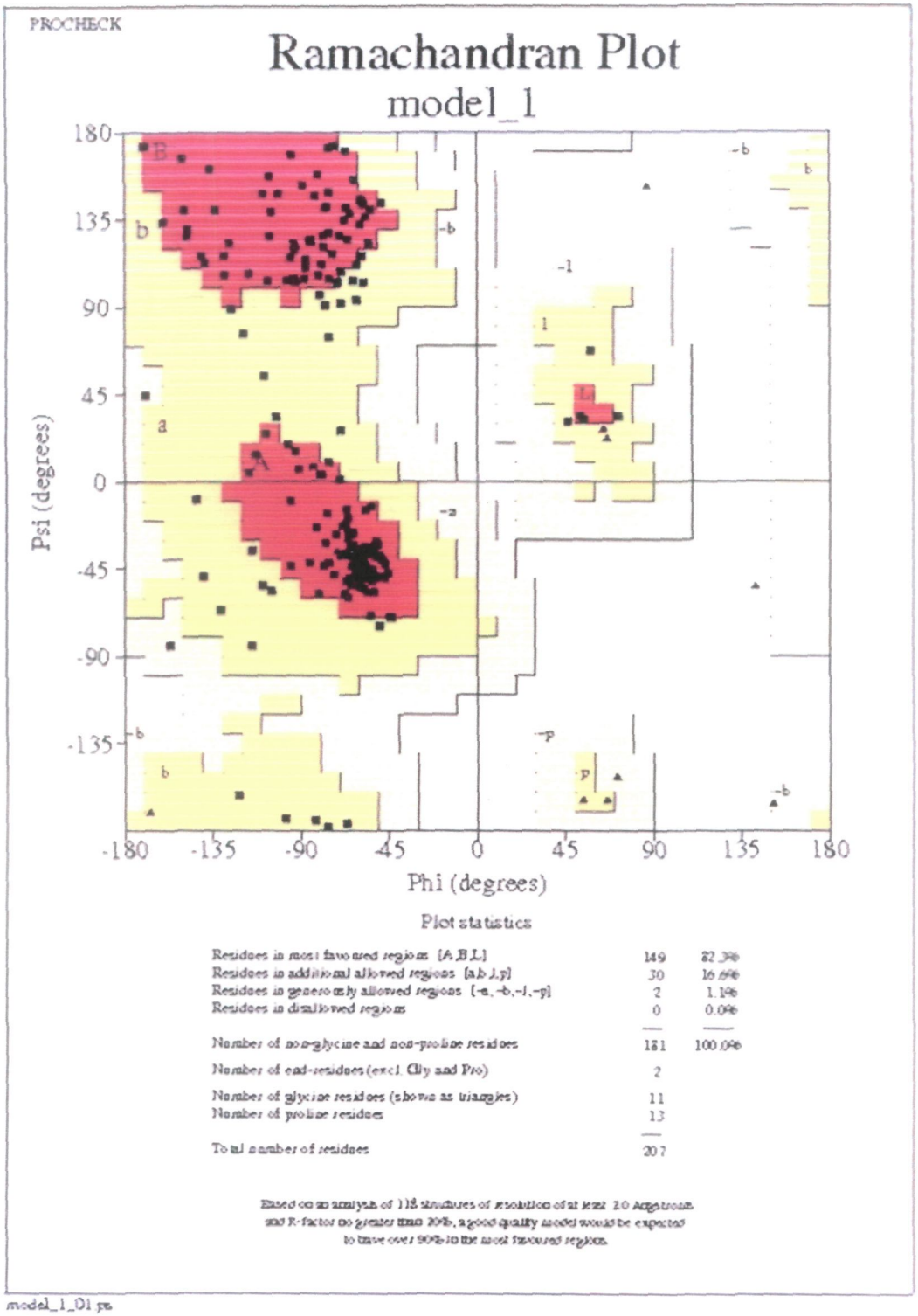


Fig.56. Ramachandran plot of the PhzD amino acid sequences of the SBJ1357 strain.

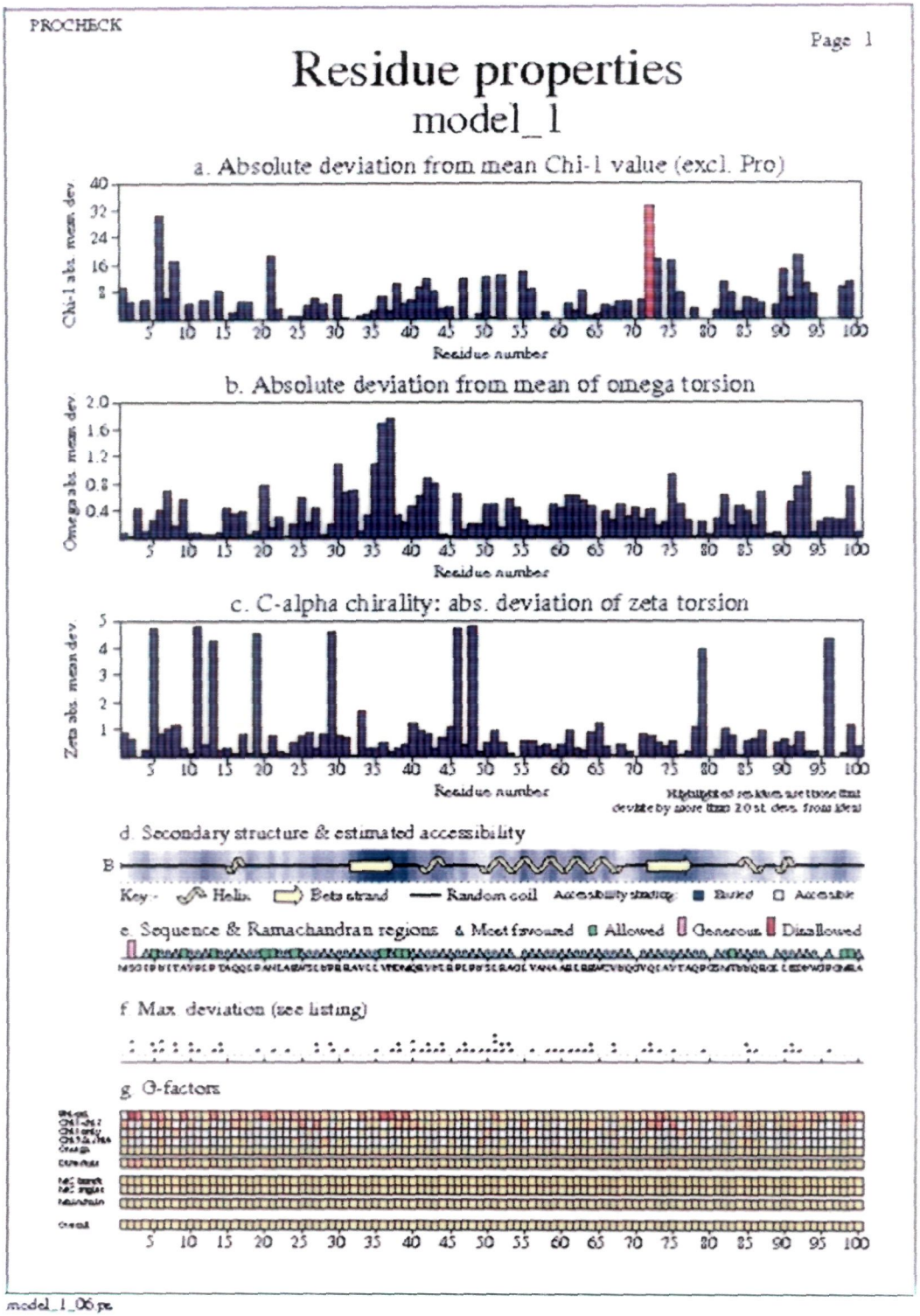


Fig.57. Comparative residues properties of the 3D PhzD protein model of SBJ 1357 strain.

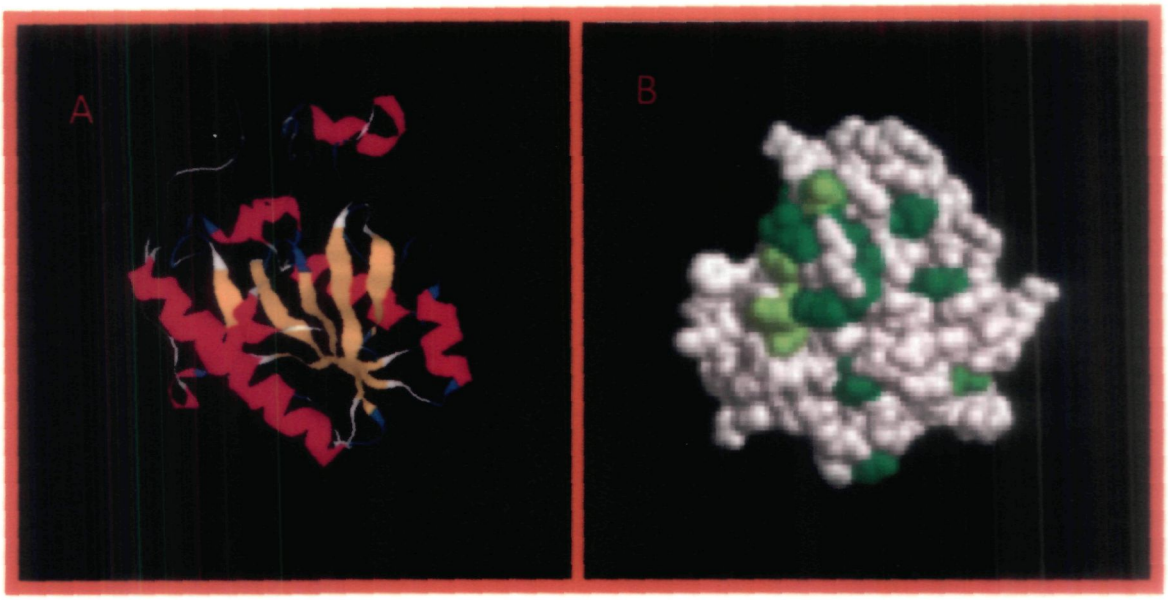


Fig. 58 (A); 3D model of the Isochorismatase hydrolase enzyme (PhzD protein) encoded by *phzD* gene of strain SBJ1357 constructed using molecular modeling. The model shows the helices, strands and turns (10, 6 and 18) using pink, yellow and blue, respectively.
(B); 3D model of the Isochorismatase hydrolase enzyme of strain SBJ1357 display the functional sites.

3.4. Plant growth promoting activities of the strain SBJ1357

3.4.1. IAA production by SBJ1357

The results shown in Fig.61 clearly demonstrate the production of significant amount of IAA in LB broth medium by SBJ1357. The estimation of IAA produce by strain SBJ1357 was carried out using the calibration curve of IAA. The prolonged incubation of culture up to 4 days showed some reduction in cell viability. However, sufficient bacterial biomass persisted in the stationary phase culture even on day 4 of incubation. The culture filtrate at different time intervals showed a linear and time dependent increase in IAA production. Enhanced production of IAA, 19.64 μgml^{-1} and 24.12 μgml^{-1} was noticed in the presence 250 μgml^{-1} and 500 μgml^{-1} of tryptophan concentration, respectively. *vis-à-vis* 9.64 μgml^{-1} IAA without tryptophan also has been detected (Fig.59). Furthermore, a linear increase in IAA production up to 4 days exhibited stability of the metabolic cells during stationary phase.

3.4.2. Inorganic phosphate solubilization by SBJ1357

The strain SBJ1357 also exhibited significant phosphate solubilization. The qualitative assays show the zone of phosphate solubilization on Pikovskaya's and NBRI-P media, respectively (Fig. 60A and B). The quantitative assay was carried out in Pikovskaya's broth and amount of soluble inorganic phosphate released in liquid medium due to solubilization of tri-calcium phosphate has to be determined 53.85 μgml^{-1} upon 5 days of growth using the calibration curve of KH_2PO_4 at 600 nm (Fig.61). The data showed the time dependent increase in the amount of solubilized inorganic phosphate, and inverse relationship with the pH of the medium. Periodic monitoring of pH of the culture filtrate revealed a significant reduction from pH 7.4 to 4.0.

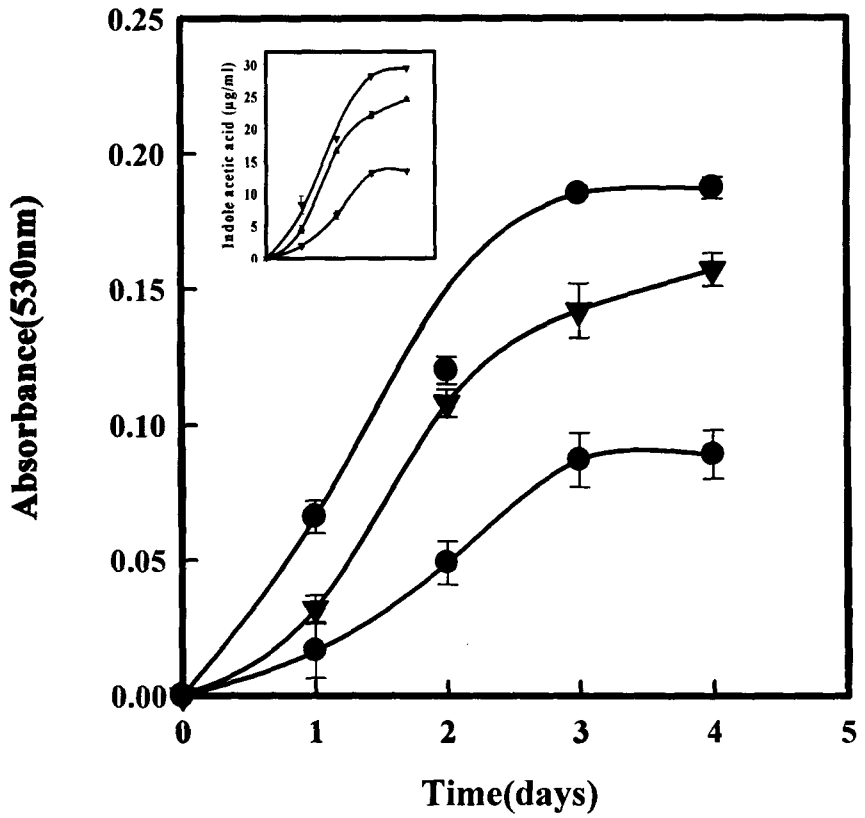


Fig.59. Quantitative estimation of IAA based on the absorbance data using the calibration curve of pure IAA at 530 nm. Strain SBJ1357 shows the production of indole acetic acid (IAA) in LB broth medium during growth of strain at different time points. Inset show the amount of IAA produced by strain SBJ1357 in the presence of tryptophan 250 μgml^{-1} and 500 μgml^{-1}.

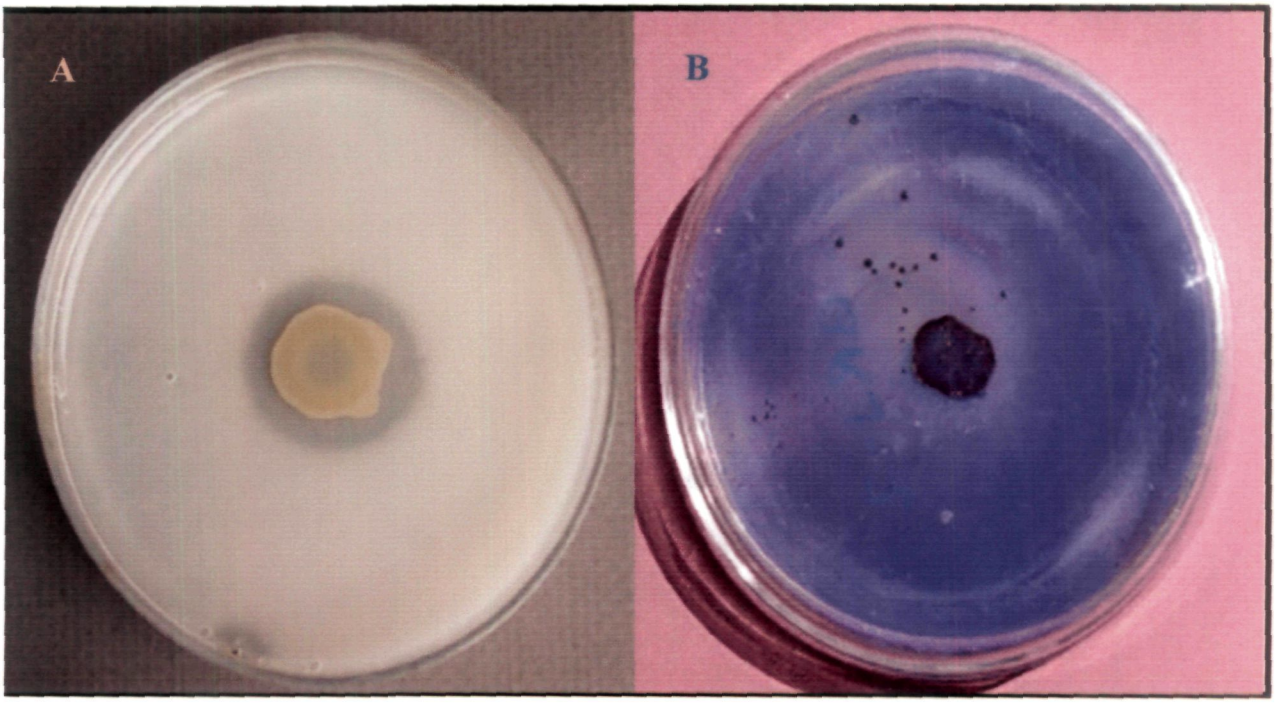


Fig.60 . (A) and (B); Qualitative assessment of phosphate solubilizing activity of strain SBJ1357 on Pikovskaya's and NBRI-P media, respectively.

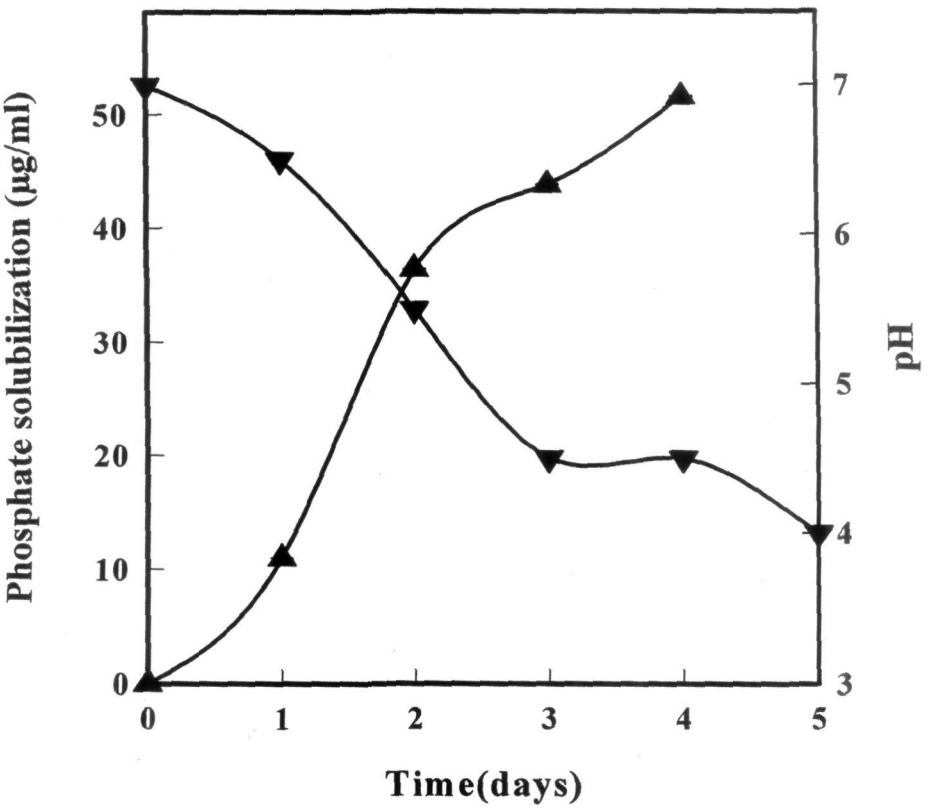


Fig.61. Quantitative assessment of inorganic phosphate as determined from the absorbance data using the calibration curve with KH_2PO_4 at 600 nm. The data obtained for strain SBJ1357 are plotted as function of time. The inset shows the pH variation in the Pikovskaya's broth medium during growth of strain at different time points.

Chapter-IV

Discussion

4.1. Discussion

The strain SBJ1357 isolated from the rhizosphere soil has exhibited multifarious biological activities. Considering the importance of valuable activities of strain SBJ1357, its characterization was carried by biochemical, Biolog and 16S rRNA gene sequence. The characterization of strain SBJ1357 validates the strain SBJ1357 as *Pseudomonas aeruginosa.* The 16S rDNA-based phylogeny is often used as reference for pseudomonad classification (Anzai *et al.,* 2000; Moore *et al.,* 1996). The microcosm study revealed the ability to used atrazine as a sole carbon and energy source. Almost complete degradation of atrazine occurred with in 20 days under microcosm conditions. The resistance to atrazine and activated atrazine degrading metabolic system in the strain SBJ1357 is due to prolonged exposure and development of strong adoptability of the bacteria in atrazine contaminated natural environment. The strain may after significance for bioremediation and cleanup of atrazine degrading soil after optimization of conditions. The bacteria can also acquire these traits through horizontal gene transfer between different bacteria under selective environmental pressure (Dai *et al.,* 2007).

The strain SBJ1357 exhibited broad spectrum antimicrobial activity and produce significant amount of siderophore. This low molecular mass biomolecules is known to act as a growth factor, and also exhibits potent antimicrobial activity (Leong, 1986; Bano and Musarrat, 2004).

The strain SBJ1357 has also demonstrated significant cyanogenic activity. The amino acid glycine acts as a metabolic precursor and stimulates HCN production (Castric, 1977), which is considered one of the factors contributing to antimicrobial activity against pathogens. The HCN regarded as an important trait for biocontrol of phytopathogenic fungi (Ellis *et. al.,* 2000; Voisard *et al.,* 1989). Indeed, Sharifi-Tehrani *et al.,* (1998) showed that HCN^+ biocontrol pseudomonads were more efficient than their HCN^- counterparts against *Fusarium* crown and root rot of tomato and *Pythium* damping-off of cucumber. Moreover, in extensive studies correlations were found between HCN production *in- vitro* and biocontrol ability of the strains in plants in two pathosystems (Ramette *et al.,* 2003).

The enzyme HCN synthase, which catalyses the formation of HCN and CO_2 from glycine, is believed to be a membrane-bound flavoprotein (Wissing and Andersen,

1981). The systematic name cyanide-forming glycine hydrogenase was proposed for HCN synthase based on nucleotide sequence data are available the database under accession numbers AJ418442 through AJ418477. The nucleotide sequence exhibited similarities with known dehydrogenases and oxidases (Blumer and Haas 2000). Although the three structural genes (*hcnA*, *hcnB*, and *hcnC*) encoding HCN synthase are reported for strain *P. aeruginosa* PAO1 (Pessi and Haas, 2000) and the fluorescent *Pseudomonas* sp. strain CHA0 (Laville *et al.*,, 1998), little is known about the polymorphism of these genes. Perhaps there is a link between HCN synthase characteristics and HCN production (Ramette *et al.*, 2001). A relationship exists between the ability of biocontrol pseudomonads to produce HCN and their biocontrol capacity (Sharifi-Tehrani *et al.*, 1998; Ellis *et al.*, 2000), but not much has been done understand to functionality of the *hcn* structural genes. Therefore, in this study the *In-Silico* functional analysis of the partial *hcn*B&C and *phz* operon genes has been carried out. The functional analysis of *hcn*B&C partial sequence revealed a conserved Shine-Dalgarno motif with identical sequences and position of the start codon, which raises the possibility of identical translation patterns. In addition, the results indicated that the two partial sequences correspond to the two main subunits of the HCN synthase. These sequences have been found to be conserved structurally or may be functionally, based on conserved features such as the transmembrane region and the essential ADP binding fold carriers (Laville *et al.*, 1998). No essential function has yet been reported for the C-terminal parts of HcnB and HcnC, whereas it is known that the N-terminal parts of both proteins display identical features important for cyanogenesis (Laville *et al.*, 1998). The *hcn*C sequences exhibits lower levels of total nucleotide diversity and synonymous substitutions as compared with partial *hcnB.* This reflects the existence of more substitutional constraints (i.e., less variation) for the essential N-terminal part of HcnC. However, other *hcn* sequences that are implicated in the regulation of HCN synthesis are not domain in this work and hence not discussed. Nevertheless, promoter region of *hcnA* contains specific regions such as the ANR box and a LuxR recognition site present in strain PAO1 are involved in transcriptional control of *hcn*ABC. Also a posttranscriptional control (via noncoding RNAs) involves the region around the *hcnA* ribosome binding site in *P. aeruginosa* (Pessi

et al., 2001). The level of conservation of these regulatory motifs in HCN^+ pseudomonads remains to be explored and established.

Identification of conditions which control PCA production will lead to a better understanding of the regulation of the biosynthesis of these secondary metabolites. This information may be crucial for optimizing biocontrol under practical conditions. Therefore, the effects of various physical factors have been investigated, on the growth and PCA production by SBJ1357. The PCA production started at the end of the exponential phase and progresses until the cells reached stationary phase. These results concur with the earlier observations indicating the induction of PCA production in stationary phase of culture (Mavrodi *et al.*, 1998), probably due to the late induction of *phz* genes encoding the key enzymes involved in PCA biosynthesis pathway. The study on carbon source utilization revealed that the glucose and fructose yielded the highest PCA levels, while moderate synthesis occurred in presence of lactose and sucrose. This corroborates well with the earlier report indicating that the PCA by *P. fluorescens* 2-79 (Slininger and Shea-Wilbur, 1995) and PCN by *P. aeruginosa* (Kanner *et al.*, 1978) is stimulated by glucose. Since the carbon sources found in plant root exudates and soil influence the type of antimicrobial metabolite being produced by biocontrol strains irrespective of their effects on bacterial growth, PCA production may differently regulate in various *Pseudomonas* spp. The results obtained with other strains are difficult to compare with our strain because of different culture conditions. Under our treatment conditions, maximum PCA production was noticed in presence of 0.5 to 1.0 mM fructose followed by 0.5mM glucose. Increasing the amount of fructose and glucose as carbon source from 0.5 to 1.0 mM resulted in reduction in PCA production. The edaphic factors that have been reported to affect PCA production are temperature (Shanahan *et al.*, 1992), soil moisture (Georgakopoulos *et al.*, 1994), and pH (Ownley *et al.*, 1992). The strain SBJ1357 also exhibited the production of PCA is severely influenced by the pH of the culture medium. The optimum production by SBJ1357 was noticed at pH 8.0 to 8.5 as compared to *P. fluorescens* 2-79, in which the optimum is pH 7.0, with only a small reduction at pH 6.0, and a severe reduction at pH 8.0 (Slininger and Shea-Wilbur, 1995). The strain SBJ1357 also showed the promising antimicrobial activity under slightly acidic conditions in comparison to the neutral pH. Chin-A-Woeng *et al.* (1998)

also suggested that pH affects the activity of the antimicrobial metabolites, and reported higher *in vitro* antifungal activity of PCA and PCN at pH 5.7. The PCA production has been completely abolished under more acidic conditions. This could be due to the repression of the *phz* operon. This is in accordance with earlier studies demonstrating the inhibition of the PCA production under more acidic conditions (Ownley *et al.,* 2003). Moreover, biocontrol by *P. fluorescens* 2-79 against take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* increases with an increasing pH (Ownley et al. 1992). This demonstrates that the pH of the rhizosphere is an important factor for successful biocontrol. The data suggests that the inconsistent performance of the biocontrol agents may be attributed to the variation in soil pH between different soil types (Ownley *et al.,* 2003). However, the nature of the phenazine derivatives may contribute to the differences in biocontrol activity between strains (Chin-A-Woeng *et al.,* 1998). Temperature is another important factor under practical plant production conditions. The results suggested optimum PCA production at 37^0C as evident from the change in yellow to green due to the accumulation of pyocyanin, (phenazine derivative). These corroborate the earlier observations indicating rapid accumulation of the pyocyanin at 37 ^{0}C (Turner and Messenger, 1986).

Similarly, the mineral effects on PCA production demonstrated the association between soil chemical and physical properties and suggested the variable performance of biocontrol strains at field sites. The data on the relationship between $ZnSO_4$ concentration and PCA production by SBJ1357 suggested the stimulatory effect. The relationship of $ZnSO_4$ with the antimicrobial metabolites production and their activity is well recognized. Ownley *et al.* (1991) reported a positive correlation between $ZnSO_4$ content in soil and the biocontrol activity of *P.fluorescens* 2-79. Also the zinc stimulated production of PCA as primary biocontrol determinant in strain 2-79 and in CHA0 strain in the naturally disease suppressive soils from where this strain was isolated. CHA0 is not effective when added to disease conducive soils that contain low amount of zinc (Defago and Haas, 1990; Slininger and Jackson, 1992).

Based on the comparisons between PCA production in SBJ1357 and other antifungal metabolites (AFM) producing *Pseudomonas* spp., it is concluded that environmental factors have the positive and negative effects on PCA production by

different strains. Therefore, it would be advisable to be aware of such effects before using any biocontrol strain. The factors affecting PCA production are likely to affect antimicrobial activity and can explain, at least partially, the inconsistency of biocontrol in field experiments. For successful biocontrol by *Pseudomonas* spp., one needs to understand that which and how the environmental factors affect the production of AFM in potential biocontrol products. Environmental gene regulation seems to differ between *Pseudomonas* spp., strains; therefore, different strains may have to be selected for different field conditions. Selection of bacterial strains that match with certain field conditions could be a strong tool in achieving successful biocontrol. Unraveling the molecular aspects that integrates PCA production is needed for successful biocontrol and will be a challenging field for future research.

The AHL production in cross feeding experiment revealed inherent presence of QS system in strain SBJ1357. The SBJ1357 strain confirmed the presence of QS system in regulating the *phz* operon responsible for synthesizing PCA. The relationship of QS signals with the PCA production is well recognized. The reporter strain used in the cross-feeding assay has the ability to detect *N*-3-(oxooctanoyl)-L-homoserine lactone and a range of its analogues (Fuqua and Winans, 1996). Previous studies have shown that *P. aeruginosa* produces *N*-3-(oxododecanoyl)-L-homoserine lactone and *N*-(butyryl)-L-homoserine lactone and that these quorum sensors are required for the expression of the genes responsible for phenazine and other compound productions (Passador *et al.*, 1993). It should be emphasized that a weak response by stains SBJ1357 and NJ101 in this assay does not mean that the test strain failed to make strong quorum sensing signals. It merely shows an inability to produce AHLs that are recognizable to *A. tumefaciens* or that the levels of the AHLs are relatively low. Thus, strain SBJ1357 and NJ101 although showed a weaker signal has suggested that the induction of PCA production with the strain SBJ1357 is AHL regulated. The presence of QS signals in the plant associated bacteria, and a few of them are plant growth-promoting and biocontrol bacteria such as *Pseudomonas aeruginosa, Pseudomonas aureofaciens, Pseudomonas putida* and *Pseudomonas fluorescens* is well known (Bassler, 1999; Chin-A-Woeng *et al.*, 2001; Pearson *et al.*, 1997; Whitehead *et al.*, 2001).

Moreover, study beside *rrs* phylogenies, the protein coding genes *hcn*BC and *phz*CDE&S in this work were compared. Although *rrs* is under stringent functional constraints (Woese, 1987), the complete or partial replacements can nevertheless occur and may result in the formation of mosaic sequences, thereby leading to a wrong organisms phylogeny in certain cases (Wang and Zhang 2000; Yap *et al.*, 1999). However, the composite approach adopted in this study appears to be appropriate and more robust the *rrs*-based species phylogeny. The *hcn* and *phz* operon based genetic relatedness together confirmed the identity of the strain. Phylogenetic analysis revealed that ability for the HCN and PCA production is an inherent trait present in strain SBJ1357. Such traits are reported in strains of deleterious pseudomonads (pathogenic to plants or animals). Interestingly, PCA^+ and HCN^+ biocontrol strains were also found clustered with phytopathogenic pseudomonads such as *P. syringae*, *P. brassicacearum*, and *P. corrugata*. This may reflect the partial overlap of their ecological habitats, the incomplete assessment of their biocontrol and pathogenic activity on different host plants. Indeed, certain pathogenic *P. brassicacearum, P. thivervalensis*, or *P. corrugata* isolates can protect wheat from take-all (Ross *et al.*, 2000; Schmidt *et al.*, 1997) or potato from *Fusarium* dry rot (Schisler *et al.*, 1997). Based on the assumption that the *rrs*-based phylogeny is the species phylogeny, the phylogeny derived from *hcnB&C* and *phzCDE&S was* found partly incongruent with the former. This may be due to genomic rearrangements and recombinations or the occurrence of lateral gene transfer. Lateral gene transfer events can lead to a distorted history between ribosomal and protein-coding genes (Ochman *et al.*, 2000). However, for biocontrol pseudomonads that produce both HCN and Phl, cluster analyses of biosynthetic genes *hcnBC* and *phlD* (Ramette *et al.*,, 2001; Wang *et al.*,, 2001) indicated that their phylogeny is not only congruent with each other but also very similar to the dendrograms based on randomly amplified polymorphic DNA data (Keel *et al.*,, 1996) and repetitive sequence-based PCR data (Mavrodi *et al.*,, 2001). Therefore, *hcnB&C* and *phzCDE&S* evolution may parallel whole genome history. However, phylogenetic analysis (*phzCDE&S*) revealed the *phz* operon of pseudomonas and some other bacterial spp. may have some ancestral relation with actinomycetes.

PCA and HCN groups were identified based on both the nucleotide and amino acid sequence analysis. The PCA and HCN groups could correspond to different

ecologically adapted populations of pseudomonads. Ramette *et al.* (2003) showed that HCN-4 strains produced less HCN overall compared with the others and group Hcn-2 did not differ from Hcn-1 and Hcn-3 in terms of HCN production, but it displayed the highest strain to strain variation in HCN production and the highest DNA diversity for both *hcn*B and *hcn*C data sets. Thus, certain differences in *hcn*BC sequences may correlate with quantitative HCN production. Although, the information on the PCA producing bacterial grouping and correlation of sequence diversity with PCA production efficacy remain to be established .However, it must be kept in mind that nothing is known about actual PCA and HCN production by the strains in their natural environment (i.e., the rhizosphere), as direct PCA and HCN measurement in situ remains a major technical issue. In the rhizosphere, carbon sources available to rhizobacteria are mainly provided by root exudates (Hale *et al.,* 1978), whose composition differs depending on the plant species. Thus, the ability to utilize particular root exudates compounds is probably an adaptation to rhizosphere habitats.

The conserved domains search of PhzC&D proteins revealed the similarity with the enzymatic (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) structure from *Mycobacterium tuberculosis*. In contrast to PhzC protein, PhzD protein of the strain SBJ1357 revealed the similarity with the enzymatic (2,3 dihydro2,3-dihyroxybenzoate synthase) structure from *Pseudomonas aeruginosa* PAO1. The data suggested the evolutionary link between the Gram +ve and –ve bacteria. The 3D structure based fold recognition study of the PhzD protein exhibited 13 % to 10 % identity and 100 % estimated precision with different Isochorismatase-like hydrolases. The Isochorismatase-like hydrolases of strain SBJ1357 also showed the structural homologies to eukaryotic hydrolases (yeast, *Caenorhabditis elegans*). 3D structure of PhzD of SBJ1357 was constructed by homology modeling using X-ray crystallographic structure of the PDB:1nf9 as a template. The 3D Model for PhzD of strain SBJ1357 was constructed and verified. The Ramachandran plot qualities do not show the amount (%) of residues belonging to the disallowed region of the plot. The molecular modeling data revealed that the conservation of the Isochorismatase hydrolases enzyme, crucial for PCA production traits among the *Pseudomonas* spp.

The auxiliary plant growth promoting activity of the strain SBJ1357 was also investigated. The data revealed the substantial production of IAA in mineral salt medium supplemented with tryptophan. The linear increase in IAA production up to 4 days exhibited stability of the metabolic cells during stationary phase. These results concur with the earlier observations indicating induction of IAA production in stationary phase of culture (Salamone *et al.*, 2001), probably due to delayed induction of a key enzyme of the IAA biosynthesis pathway. Several plant growth promoting rhizobacteria are known to secrete IAA into culture media, and have been shown to stimulate plant growth (Wang *et al.*,1982; Barbieri *et al.*, 1986; Gaudin *et al.*,1998; Bano and Musarrat, 2003, 2004; Zadi *et al.*, 2006).

Beside the IAA production, formation of a clear zone on Pikovskaya's medium unequivocally suggests the phosphate solubilizing potential of the isolate. Quantitative analysis confirmed the release of soluble phosphate from tricalcium phosphate in the medium with a significant transition in pH of the medium from, due to acid production. This is in accordance with earlier studies demonstrating the production of organic and inorganic acids by phosphate solubilizing microorganisms (Pareek and Gaur, 1976; Bano and Musarrat, 2003). Thus, substantial productions of IAA and phosphate solubilization by the isolate SBJ1357 clearly suggest its inherent plant growth promoting potential.

In conclusion an agronomically important bacterial strain *Pseudomonas aeruginosa* SBJ1357 has been isolated. A composite system determining phylogenetic relatedness based on rRNA, HCN and PCA encoding genes was tested. The *In-Silico* analysis and molecular modeling of provide the information on the functionality and evolution of the related genes. In spite of heterogenecity or polymorphism of Hcn and Phz proteins, the fold molecular modeling studies exhibited the commonality in structure and function even with the distant organisms. The distant relatedness suggests the natural molecular austerity of the biological systems. The molecular modeling also suggested the different gene products with similar 3D configuration with conserved domains/motifs may exits in distantly related organisms, which belong to the same family and perform similar functions. These activities of the strain SBJ1357 could be utilized for the environmental, pathological and agronomic purpose. Further extensive study on the regulation of these traits of strain SBJ1357 is warranted.

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Appendices

Appendix 1: Minimal salt medium (MS-medium)

	(gl-1)
Ammonium sulphate	1.00
Dipotassium hydrogen orthophosphate	1.00
Disodium hydrogen orthophosphate	2.10
Magnesium sulphate	0.01
Calcium chloride	0.10
Ferric chloride	0.001
Copper sulphate	0.040
Sodium molybdate	0.002
pH	7.2±0.2

(For the preparation of solid medium, 1.5% (w/v) agar-agar was added to the above solution)

Appendix 2: Potato dextrose agar medium

	(gl-1)
Glucose	40.0
Peptone	10.0
Agar-agar	15.0
Ph	7.0 ± 0.2

Appendix 3: Gram staining

Primary stain	
Solution A	
Crystal violet	2.0 g
Ethyl alcohol (95%)	20.0 ml
Solution B	

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B were then mixed.

Gram's iodine	
Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 ml

Ethyl alcohol (95%)	
Ethyl alcohol	95.0 ml
Distilled water	5.0 ml

Counter stain	
Safranin–O	2.5 g
Ethyl alcohol (95%)	100.0 ml

(Dilute 10 times with distilled water before use)

Appendix 4: Nutrient broth

	(gl-1)
Beef extract	1.5
Yeast extract	1.5
Peptone	5.0
NaCl	5.0
pH	7.2±0.2

Appendix 5: Kovac's Reagent	g/l
p-dimethyl aminobenzaldehyde	5.0
amyl alcohol	75.0
Hydrochloric acid (concentrated)	25.0

Appendix 6: Voges-Proskauer test

Solution A	
Alpha-naphthol	5.0 g
Ethyl alcohol (absolute)	95.0 ml
Solution B	
Potassium hydroxide	40.0 g
Creatinine	0.3 g
Distilled water	100.0 ml

(Potassium hydroxide was dissolved in 75 ml of distilled water followed by the addition of creatinine and the final volume was made upto 100 ml. The solution was stored at 4oC)

Appendix 7: Methyl red solution

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

(Methyl red was dissolved in 95% ethyl alcohol and diluted to 500 ml with distilled water)

Appendix 8: Barrits Reagent

Solution A	
α-napthol	5.0
Ethanol (absolute)	95 ml

Solution B

Potassium hydroxide	40.0
Creatinine	0.3
Distilled	100 ml

Appendix 9: Simmon's citrate Agar

Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Magnesium sulphate	0.2
Bromothyl blue	.08
Agar	15.0
pH	7.2

Appendix 10: Nutrient agar

Beef extract	1.5
Yeast extract	1.5
Peptone	5.0
NaCl	5.0
Agar	15.0
pH	7.2

Appendix 11: Nitrate broth

	gl-1
Peptone	5.0
Beef extract	3.0
Potassium nitrate	5.0
pH	7.0±0.2

Appendix 12: King's B Agar

Protease peptone	20.0
Dipotassium hydrogen orthophosphate	2.5
Glycerol	10.0
Agar	20.0
pH	7.2

Appendix 13: Luria Bertani Broth

Peptone	10.0
Yeast extracts	5.0
Sodium chloride	4.0
Agar	15.0
pH	7.2

Appendix 14: Pikovskaya's agar medium

	(gl-1)
Tri-calcium phosphate	5.00
Sucrose	10.0
Ammonium sulphate	0.50
Sodium chloride	0.20
Manganese sulphate	0.10
Potassium chloride	0.20
Yeast extract	0.50
Mangnese sulphate	Trace
Ferrous sulphate	Trace
Agar-agar	15.00
pH	7.0±0.2